Fps1p channel is the mediator of the major part of glycerol passive diffusion in *Saccharomyces cerevisiae*: artefacts and re-definitions

Rui Oliveira, Fernanda Lages, Magda Silva-Graça, and Cândida Lucas*

Department of Biology /Environmental Sciences Research Centre (CCA/B)

University of Minho, Campus de Gualtar, 4710-057 Braga, Portugal

Key words:

S. cerevisiae, glycerol transport/permeability artefacts, *FPS1*, *YFL 054c*, *GUT1, GUT2*

Corresponding author.

Phone: 351-253 604313/10; **Fax**: 351-253678980; **E-Mail**: clucas@bio.uminho.pt

Abstract

Glycerol has been shown to cross *Saccharomyces cerevisiae* plasma membrane (1) through a H^+/symport detected in cells grown on non-fermentable carbon sources, (2) by passive diffusion and (3) through the constitutive Fps1p channel. This has been named a *facilitator,* for mediating glycerol low affinity transport of the facilitated diffusion type. We present experimental evidence that this kinetics is an artefact created by glycerol kinase activity. Instead, the channel is shown to mediate the major part of glycerol passive diffusion. Nevertheless, Fps1p major role *in vivo* has been previously shown to be the control of glycerol export under osmotic stress or in reaction to turgor changes. Overexpressing *FPS1* caused an increase in H^+/symport V_{max}. Moreover, *yfl054c* and *fps1* mutants were equally affected by exogenously added ethanol, being the correspondent passive diffusion stimulated. This is, to our knowledge, the first time that a phenotype can be attributed to the functioning of *YFL054c* gene. Glycerol passive diffusion is thus apparently channel mediated. This is discussed according to glycerol chemical properties, which contradict the widely spread concept of glycerol *lipossoluble nature*. The discussion considers the multiple roles that glycerol intracellular levels and its pathway regulation might play as a central key to metabolism control.

1. Introduction

In the yeast *Saccharomyces cerevisiae*, glycerol plays important roles in metabolism, from which the most studied is the one of compatible solute, accumulated under osmotic stress conditions. A lot of information is available on how the genes from glycerol pathways respond to osmotic stress signalling [1,2,3,4,5], regulating the production of this substrate in accordance to the degree of stress the cells are subjected to. Despite this, a retention problem remains to be completely clarified, essentially due to the so-called *liposoluble nature* of this compound [6,7]. Accordingly, glycerol should leak through the plasma membrane, regardless of the environmental conditions. This has been accepted as common knowledge among the yeast scientific community, somehow hampering the acceptance of the existence of proteins able to permeate and control glycerol movements across the yeast plasma membrane.

According to previous results [8,9], glycerol is actively transported by a proton symport in cells cultivated in non-fermentable carbon sources, like ethanol, glycerol or acetate. It is subjected to glucose regulation, being undetectable in repressed cells by growth on glucose [8]. These, instead, take up glycerol through Fps1p, described as a low affinity transport of the facilitated diffusion type [10]. The basis for this conclusion lies essentially in the fact that transport did not induce glycerol intracellular accumulation against its chemical gradient and was not affected by the action of an ionophore [10]. According to the same authors, mutants defective on *FPS1* gene did not display this uptake, reason why it was attributed to Fps1p activity.

The *FPS1* gene codes for a channel-type protein of the MIP family, despite the rather unusual topology with two long terminal hydrophilic extensions [11,12,13,14]. The

main physiological role of Fps1p, according to M. Tamás and collaborators [13], is to regulate glycerol export under sudden osmotic down-shock, reacting to changes in cell turgor, rather than glycerol uptake. Yet, the channel closes upon osmotic up-shock, and can thus contribute to immediate glycerol retention [11,13]. Another ORF, with high degree of homology to *FPS1* gene [15,12], *YFL054c*, has been identified, but no related phenotype has been described so far [16,15,1], although both genes have been shown to be genetically close to aquaglyceroporins [12].

The physiological characterisation of *S. cerevisiae* glycerol H^+-symport was performed using a diploid wild type strain from the YPCC (IGC3507), cultivated in MM and collected in mid exponential growth phase [8]. Under these strict growth conditions, the low affinity glycerol activity attributed to Fps1p was not detected. On the contrary, *fps1* mutants (from W303-1A genetic background), grown under derepression conditions, using either ethanol or glycerol as carbon and energy sources, presented active glycerol H^+/symport, without further detectable second order kinetic uptake component [10]. This way, it was possible to establish that one and the other types of transport were unrelated, differently regulated and most likely used by the cell for different purposes [10].

This work presents evidence that Fps1p channel does not mediate transport of a saturable nature, being the facilitated diffusion previously described [10], dependent on the presence and activity of glycerol kinase, encoded by *GUT1* gene. The interference of this enzyme on glycerol uptake measurements has been first described in *Escherichia coli* [17]. More recently, B. Hölst and co-workers [18] also described in *S. cerevisiae* this transport artefact, apparently caused by enzyme-driven substrate saturation, when the *GUP1* and *GUP2* genes were identified as putative glycerol transporters.

Further experimental data aims to clarify the relationship between Fps1p, mediated glycerol entry and passive diffusion changes according to cell physical reaction to stress. A weak phenotype for *YFL054c*, suggesting that it can also mediate glycerol entry, is shown for the first time. Concomitantly, the long-time accepted concept of glycerol *liposoluble nature* is re-visited and re-analysed.

2. Materials and Methods

2.1. Yeast strains and growth conditions

S. cerevisiae strains, listed in Table I, were maintained at 4°C in YNB w/o amino acids (Difco) supplemented with 2% (w/v) glucose and amino acids, according to the strains demands, or YEP (peptone, 2%w/v and yeast extract, 1%w/v) supplemented with 2% (w/v) glucose (YEPD) and at –70°C in glycerol 30% (w/v). Batch cultures were performed using YNB (Difco) (MM) or complete medium (YEP) supplemented with 2% (w/v) glucose or 2% (v/v) ethanol as carbon sources. Incubation conditions were standardised at 30°C and 180 rpm orbital shaking, in 500 ml Erlenmeyer flasks containing 200 ml of growth medium. Growth was followed spectrophotometrically at A_{600nm}.

2.2. Measurements of glycerol uptake

Glucose- or glycerol-grown cells were harvested by centrifugation, washed twice and resuspended to a final concentration of ±30 mg dry wteight ml^{-1} in ice-cold distilled water. Initial uptake rates of glycerol were determined as described before [8], using [^{14}C]glycerol in ethanol-free solutions, ranging from 0.2 to 40 mM, with variable specific activity (s.a.), respectively, from 2800 to 250 dpm $nmol^{-1}$. Computer regression analysis program GraphPad PRISM^{R} (1994-97 Copyright GraphPad Software, Inc.) was used to determine transport kinetic parameters and evaluate their statistical validity.

2.3. Measurement of intracellular volume

The intracellular volume was measured as previously described [19,20]. Measurements were performed in the absence and in the presence of sodium chloride in the reaction buffer using different incubation periods.

2.4. Measurement of [^{14}C]-glycerol accumulation

To measure [^{14}C]glycerol accumulation, the same method described before was used [21]. The experiment was started by the addition of 10 mM [^{14}C]glycerol (s.a. ± 700 dpm nmol^{-1}). A parallel experiment was performed, adding to the reaction buffer from the start of the experiment, 50 μM of the ionophore CCCP (carbonyl cyanide m-chlorophenyl hydrazone). The capacity to induce efflux of intracellular accumulated radiolabelled glycerol was assayed with 50 mM *cold* (non-radioactive) glycerol.

2.5. Measurements of intracellular and extracellular compounds

Compounds identification and quantification were performed by chromatography (HPLC), using the same methodology applied before [8].

2.6. Enzymatic assays

Cell free extracts were prepared according to L. Neves and collaborators [22]. Glycerol kinase activity and total protein concentration determinations were done according to the methodology described previously [18].

2.7. Mutant strains integrity control

The integrity of the strains interrupted in *GUT1, GUT2* or *FPS1* was controlled periodically, confirming the correspondent auxotrophic marks, as well as testing PCR amplification of the deleted strains DNA with specific primers for each gene (not shown). Furthermore, the *fps1* mutant strains were assayed as to arsenite and antimonite survival ability in comparison to parental strain W303-1A, according to R. Wisocki and collaborators [23] (see Acknowledgements).

2.8. Northern analysis of FPS1 expression

For RNA isolation, cells were harvested by centrifugation in mid-exponential growth phase (OD_{600} 0.4 - 0.5) and during diauxic-shift (OD_{600} 1.1 - 1.2), and washed twice with ice-cold distilled water. RNA was isolated with hot acidic phenol according to standard procedures [24,25,26]. Probes for *FPS1* and internal standard, rRNA 18S, were obtained by PCR, using genomic DNA as template for *FPS1* and cDNA for internal standard. Primers designed for *FPS1,* were: forward, $^{5'}$CCTACAGTCTTGCCCTCCAC$^{3'}$, and reverse, $^{5'}$AACATTCCCGCAACACTTTC$^{3'}$. Primers designed for internal standard were: forward, $^{5'}$AGGAATTGACGGAAGGGCAC$^{3'}$, and reverse $^{5'}$GGACATCTAAGGGCATCACA$^{3'}$. Probes were labelled with alkaline phosphatase with CDP-Star™ (Amersham Pharmacia Biotech) for subsequent chemiluminescent detection with CDP-Star™ detection reagent. Membranes were revealed using Hyperfilm™ ECL. Images were digitalised and densitometric analysis was performed using NIH Image 1.60 software.

2.9. Ethanol effect over glycerol entry

Cells were cultured as above mentioned in YEPD, up to mid-exponential growth phase, ≈ 0.5 OD_{600}. Uptake was measured incubating the cells for 5 min at 30°C in Tris-citrate buffer adjusted to pH 5.0 and containing 12% (v/v) ethanol (reagent grade). Uptake was performed using ethanol-free [^{14}C]glycerol solutions, ranging from 10 to 60 mM, with variable s.a. ranging, respectively from ±3900 to ±300 dpm $nmol^{-1}$. Diffusion constants (Kd) were calculated using computer regression analysis software Graph Pad PRISM^{R} as mentioned above.

2.10. Reproductivity of results

All the transport and enzyme assays were repeated at least three times, and the data reported are either average or representative values. The exact number of experiments was mentioned whenever considered relevant.

3. Results

3.1. Glycerol transport studies in MM glucose-grown cells

The wt strain IGC3507 was cultivated in MM with glucose and collected along the growth curve, until diauxic-shift, when glucose had been reduced to less than 0.02% (w/v) and considerable amounts of ethanol were present in the medium. In spite of the

partial derepression that happens during the metabolic shift, glycerol mediated uptake was not detected. Instead, non-saturable uptake first-order kinetics was measured during diauxic shift (not shown), identical to the passive diffusion constants (Kd) determined before in exponentially growing cells [8]. These results were identical to the ones found previously in W303-1A, as well as in the correspondent *fps1* mutant [10]. This way, we can state that in wt repressed cells, cultivated in MM with glucose, experimental results fitted a first order kinetic equation. This was also true for cells collected in diauxic-shift phase (not shown).

3.2. Glycerol transport studies in YEPD-grown cells

According to the results presented by F. Sutherland and co-workers [10], glycerol uptake in YEPD-grown W303-1A cells presented a saturable Michaelis-Menten kinetic component, together with a first-order kinetic branch, correspondent to passive diffusion. Experimental results should thus fit the following equation:

$$V_{app} = Vmax.[S] / (Km + [S]) + Kd.[S] \quad (1)$$

The mediated uptake was attributed to Fps1p and classified as facilitated diffusion. Identical results were found in IGC3507 [8]. Nevertheless, the glycerol uptake saturable kinetics of low affinity was only detected in cells collected during diauxic-shift. Experimental raw Michaelis constants were: Km 24.6 ± 3.6 (3) mM and Vmax 288.4 ± 77.3 μmol h^{-1} g^{-1} dry weight (Fig 1). Iteration by computer regression analysis, transformed these into: Km of 7.7 ± 2.2 mM and Vmax 91.1 ± 30.9 μmol h^{-1} g^{-1} dry weight, and suggested the existence of a non-saturable branch with a Kd of 0.003 ± 0.001 l h^{-1} g^{-1} dry weight. Km was higher than the one determined for glycerol

proton symport but V_{max} was considerably low. Kd presented also a value lower than the ones measured before, ≈ 0.005 to 0.006 l h^{-1} g^{-1} dry weight [8]. These results indicated the existence of a facilitated diffusion for glycerol, although exclusively detectable in cultures in fermentation/respiration transition phase.

Thus considering, the results are kinetically similar to the ones published on W303-1A [10] and fit the model first suggested by K. Luyten and collaborators [11], consisting of a Fps1p-mediated glycerol entry together with passive diffusion and obeying Eq. 1. The only significative difference lies in Kd values: 0.013 l h^{-1} g^{-1} dry weight, in W303-A [10] and 0.003 l h^{-1} g^{-1} dry weight in IGC3507 (above). The contribution of Kd to V_{app} measurements, using Eq. 1, is increasingly higher as higher substrate concentrations are used. This has a direct effect on uptake kinetic constants determinations. Above a glycerol concentration threshold, around 50mM, the contribution of passive diffusion velocities to V_{app} overcome mediated uptake velocities, turning very difficult to discriminate mathematically the first component in Eq. 1, even through computer regression analysis.

3.3. Glycerol transport studies in salt-shocked MM glucose-grown cells

Transferring MM glucose-grown cells to the same medium with increasing NaCl concentrations, resulted, as expected, in a proportional increase in intracellular accumulated glycerol (Fig 2). This accumulation, is at first hand the consequence of an increase in glycerol production, due to *GPD1* and *GPP2* transcription stimulation by salt stress [1,2,4], through specific signal transduction pathways [3,27]. But, on the

other hand, this result must be also a consequence of an increase in glycerol retention capacity. No mediated glycerol active transport was detected in these cells, until a 3 hours maximum incubation in 1M NaCl. The increase in glycerol retention, could either be a consequence of: (i) a reduction in passive diffusion, since it reduces to ± 1/3 when cells are shocked with 1 M NaCl [8] and/or (ii) the closing of Fps1p channel [13] constitutively expressed [16,11].

Nevertheless, this could, besides other possible factors, also be an indirect consequence of cell shrinking. Cell volume decrease is one of the most direct and visible changes of the cell after an osmotic shock [28,29,21,30,9,31]. Cell shrinkage is supposed to interfere with membrane proteins stability and membrane fluidity. The degree of decrease of *S. cerevisiae* intracellular volume in relation to the degree of salt stress has been reported before for IGC3507 strain [8,9]. Using W303-1A, results were similar (not shown).

Taking into consideration that Fps1p channel closes upon an hyperosmotic salt shock, reducing glycerol uptake to ± 20 to 30 % the transport value measured before the shock [13], we measured cell volume reduction in identical experimental conditions. Cells were shocked for 2 min in 500 mM and 1 M NaCl. Volume was reduced to, respectively, 70% and 40%. We compared these results with glycerol passive diffusion Kd changes, again under the same experimental conditions. Kd measured in 1 M salt-shocked cells reduced from 0.005 ± 0.001 l h^{-1} g^{-1} dry weight (n=5) to 0.002 ± 0.0004 l h^{-1} g^{-1} dry weight (n=3) (≈ 40%). Therefore, the percentage of reduction of *S. cerevisiae* cell volume is identical to the percentage of reduction of passive diffusion Kd under the same experimental conditions.

In view of these results, glycerol transport in cells subjected to salt shock had to be better verified since, as stated in the Introduction, this relates with the main function attributed to Fps1p as a channel, i.e. regulating glycerol efflux by closing under salt-up-shock conditions or according to turgor changes. Moreover, being aware that MM is a very stringent medium, and also bearing in mind that the previously published results concerning Fps1p kinetic properties were obtained cultivating the cells in rich medium [10], we re-evaluated glycerol uptake properties in cells cultured in YEPD with salt.

3.4. Glycerol transport studies in salt-shocked YEPD-grown cells

Glycerol uptake was assayed in cells submitted to 2 min salt-shock using 0.5 and 1 M NaCl. Glycerol concentrations chosen to assay transport were 6, 14 and 50 mM. The reduction of uptake after salt shock was increasingly higher as the contribution of passive diffusion to total glycerol uptake according to Eq. 1 increased (Fig 3). The degree of uptake reduction was maximum (40%) at 1 M NaCl, using 50 mM glycerol. This confirmed that non-saturable uptake is more sensitive to salt stress.

At this point, results suggest that retention could be, at least in part, obtained through a decrease in passive diffusion, as a consequence of cell shrinkage. This way, the Kd measured after salt-shock is supposed to be the remaining glycerol entry, which is not Fps1p-mediated, since the channel should close at a sub-minute time scale under such circumstances [11,13].

Using the values in Figure 3, it was possible to calculate different Km_{app} constants for glycerol uptake at 0, 0.5 and 1 M NaCl, respectively, 21.6, 16.6 and 13.7 mM, decreasing linearly with increasing salt concentration (Fig 3/*Insert*). These

apparent Km values depend on the substrate concentration range used experimentally. If these are high enough, passive diffusion variation creates illusive high Km values. On the other hand, apparent Km values decrease with increasing salt shock, which agree with the fact that passive diffusion is the most salt-affected component of transport in Eq. 1.

At this point, we may state that, in IGC3507 repressed cells by growth on glucose, both in MM and YEP, glycerol uptake displays only non-saturable kinetics, characteristic of passive diffusion, not affected by growth medium composition, but affected by the presence of salt. Cells collected in diauxic-shift, and thus partially derepressed, present a low affinity saturable kinetic branch for glycerol entry, which, unlike passive diffusion, is not affected by salt stress, but depends on growth media stringency, since it could be detected in YEP but not in MM.

The low affinity uptake kinetics could (i) be a glycerol kinase mediated artefact as demonstrated before [13] or (ii) be caused by Fps1p closing and opening, which, at long-term could be a more dynamic process than predicted till now. The most straightforward interpretation of these results would be that the so-called Fps1p-mediated facilitated diffusion might be under glucose regulation, explaining why it could only be detected in the end of exponential growth phase in YEPD-grown cells. Yet, *FPS1* is constitutively expressed [16,11] and should be constitutively opened in the absence of salt [13]. Furthermore, if Fps1p is supposed to be closed under salt-stress, the correspondent facilitated diffusion should be measured only in the absence of salt, which is not the case (Fig 3). Furthermore, glycerol entry in ethanol-grown cells, *i.e.* under derepression conditions, should then fit a complex, three component, equation:

$$V_{app} = \left(Vmax_{\mathbf{1}}.[S] / (Km_{\mathbf{1}} + [S])\right) + \left(Vmax_{\mathbf{2}}.[S] / (Km_{\mathbf{2}} + [S])\right) + \left(Kd.[S]\right) \qquad (2)$$

This corresponds to two mediated transport systems: H^+/symport (**1**) and facilitated diffusion (**2**), plus a first-order component, passive diffusion. This is not compatible with the experimental results obtained on ethanol-grown cells [8,10]. Also, computer regression analysis does not accept as statistically valid a three component kinetic equation to fit experimental results (not shown), while statistical confidence applying Eq. 1 to the same results lies around 95%.

Taking altogether the results and rationales above, we can suggest that the so-called Fps1p-mediated facilitated diffusion might be an experimental artefact of uptake measurements.

3.5. Glycerol kinase interference in uptake measurements

We measured glycerol kinase levels of activity in IGC3507 cells collected along growth in MM-glucose and YEPD (Fig 4). Additionally, we also measured glucose consumption and extracellular accumulation of glycerol and ethanol (Fig 4). More glycerol was found in cells cultivated in MM, but glycerol kinase levels were much higher in YEP, regardless to growth phase (Fig 4 A/B). Ethanol levels in late exponential phase were rather similar and glucose disappeared from the medium at a similar rate in both media. These results show that glycerol kinase activity is not as strictly regulated by glucose as formerly thought [32], and that growth in less strictly repressed conditions, though in the presence of glucose (YEPD), can induce significative levels of this enzyme activity (Fig. 4). We can conclude, at this stage, that there is a coincidence between high glycerol kinase levels and the measurements of the supposed Fps1p-mediated facilitated diffusion.

3.6. Re-evaluation of glycerol uptake in fps1 *mutants*

In spite that it has been published that mutants defective on *FPS1* do not present facilitated diffusion [11,10], and taking into account the results presented above, the involvement of the *FPS1* gene on glycerol uptake was re-analysed. The experimental approach aimed to confirm the absence of facilitated diffusion activity on *fps1* mutants published before [10].

When cells were cultivated in YEPD and collected during diauxic-shift, both W303-1A and the correspondent *fps1* strain presented, as IGC3507, the low affinity transport mentioned above (Fig 5). *Inserts* in Fig. 5 compare the levels of glycerol accumulation, in/out ratios, obtained on glucose diauxic-shift cells with ethanol-grown cells, *i.e.* cells taking up glycerol actively. As it can be seen, diauxic-shift cells do not overcome in/out equilibrium, while ethanol-grown cells accumulate high amounts of glycerol against its chemical gradient, according to the presence of the active transporter. Ethanol-grown *fps1* mutant cells present a ± 35% higher accumulation ratio than the parental strain, though neither strain reached the values obtained by IGC3507 [8] and although no result so far indicated that the interruption of *FPS1* gene could interfere with active transport kinetics [10].

Furthermore, *fps1* mutant YEPE-grown cells were tested for the uncoupling effect of CCCP over glycerol uptake, like done before for W303-1A [10], using a rather broad substrate concentration range, which covers active uptake range and passive diffusion substrate range (Fig. 6). As before [10], the highest the contribution of passive diffusion to global glycerol uptake the lowest the uncoupling effect of CCCP. This decreased in the inverse proportion to glycerol concentrations used to measure uptake, which, as

mentioned above, correspond to increasing contribution of passive diffusion. The effect observed on *fps1* mutant was identical to wild type cells in the substrate range of active transport, but stabilised around 60% inhibition for the higher substrate range. This indicates that, in these mutants, in opposition to wild type cells, passive diffusion does not contribute to glycerol V_{app}.

3.7. Glycerol kinase measurements in fps1 *mutants*

The levels of glycerol kinase activity were determined in glucose-growing cells of *fps1* mutant (obtained from W303-1A), comparing the parental strain with *gut1* and *gut 2* mutants from the same genetic background. For control, the enzyme activity was measured in ethanol and glycerol exponentially growing cells of W303-1A: 61 to 68 mU mg protein^{-1}. Enzyme activity levels were higher, in any growth phase, in *fps1* mutant than in the parental strain (Fig. 7). During diauxic-shift, these were approximately half the activity measured in ethanol- and glycerol-grown cells. The cell samples collected to measure kinase activity were also used to measure *FPS1* expression by Northern analysis (Fig. 7, *Insert*).

Using microarray technology, *FPS1* mRNA levels have been reported to be constant along growth on glucose [33]. Although experiments were done under similar growth conditions, a different strain was used. Our results confirmed that in W303-1A expression of *FPS1* maintained identical values along growth on YEPD. Therefore, these results indicate that the saturable kinetics, characteristically detected at diauxic-shift growth phase, is not the result of an eventual increase in Fps1p activity as a consequence of an increase in *FPS1* expression.

According to F. Sutherland and co-workers [10], *fps1* strain was grown overnight and subsequently collected for assay. Although wild type and mutant strains lag phase duration on YEPD are rather similar, specific growth rates (μ_g) are very different (Fig. 8). *fps1* has a higher specific growth rate than wild type, being able to reach stationary phase while the parental strain is still in late exponential phase or around diauxic-shift. In the presence of salt, although the growth rates decrease and the lag phase duration increase as expected, the differences are considerable. The mutant lag phase is half the one of the parental strain (Fig. 8). After a 24 h growth, wild type and *fps1* present, respectively, OD_{600} of 1.4 and 3.0. This might justify why we present now so different results for glycerol uptake in *fps1* mutant than the ones published before [10]. Another factor that might have an impact on results, although we did not explore this question, is the difference in the liquid/air ratio used during cell growth: 1/4 by F. Sutherland and co-workers [10] and 1/2.5 in this work.

At this point, we observe a coincidence between (i) growth media (YEPD), (ii) growth phase (diauxic-shift), (iii) high glycerol kinase levels and (iv) the measurements of the so-called facilitated diffusion attributed to the functioning of Fps1p. Moreover, in opposition to the published results [10], *fps1* mutant presented low affinity glycerol uptake, identical to the parental strain, when cells were collected in identical growth phase.

3.8. Uptake measurements in gut1 *and* gut2 *mutants*

We measured glycerol uptake in YEPD-grown cells up to diauxic-shift using mutants defective for glycerol kinase, Gut1p, or mitochondrial glycerol 3-phosphate dehydrogenase, Gut2p, all derived from W303-1A genetic background (Table II). We

compared diauxic-shift glucose- (Fig. 9 A) with ethanol-grown cells (Fig. 9 B). The results clearly distinguish the two strains by the disappearance of the facilitated diffusion in the *gut1* strain when grown on glucose. Diauxic-shift *gut1* cells lost the saturable component and kept passive diffusion. Furthermore, glycerol accumulation capacity did not exceed equilibrium and the uncoupler CCCP had no effect on in/out ratio (Fig. 9 A *Inserts*). Instead, *gut2* diauxic-shift cells presented the saturable low affinity component and a slightly higher accumulation ratio than *gut1* mutants, not significantly different from equilibrium and also not significantly affected by CCCP.

Furthermore, ethanol-grown cells of both mutants presented the biphasic kinetics characteristic of Eq. 1 - an active transport plus a non-mediated component (Fig 9 B). Accumulation ratios varied, once more, in a way compatible with the correspondent deletions (not shown). *gut1* mutant accumulated less than *gut2* mutant, but glycerol was 100% free inside the cell and label efflux was total. In *gut2* mutant, efflux was partial, eventually due to glycerol phosphorilation and deviation to other pathways. In both cases, as expected, CCCP prevented accumulation, acting over proton motive force and thus over proton symport work (Fig 9 B *Inserts*).

We can state that all the results presented so far strongly sustain the hypothesis of the so-called facilitated diffusion being an experimental artefact created by glycerol kinase substrate saturation.

3.9. The effect of the presence of multiple copies of FPS1

Both YSH 6.36-3B and W303-1A parental strains (Table I) were transformed with the multicopy plasmid YEplac195 containing the *FPS1* gene, and used to measure glycerol active transport. These strains were grown on ethanol or glycerol and collected in mid-exponential growth phase, presenting, as expected, glycerol H^+/symport activity. Kinetic constants from radiolabelled glycerol uptake as well as glycerol driven proton uptake, and the correspondent stoicheiometry (Table III) were similar to before [8] but Vmax was ± 3 to 4 times higher than in parental strains or even in the previously used wt IGC3507 (Table III, Fig. 10 A). On the other hand, passive diffusion Kd approximately doubled: 0.027 ± 0.006 l h^{-1} g^{-1} dry weight (n>3).

Strains over-expressing *FPS1*, growing on MM with glucose and harvested in mid-exponential growth phase, unlike wild type cells, showed a transient alkalinization upon glycerol addition, although very small and thus not quantifiable, as well as accumulation of radiolabelled glycerol against gradient as exemplified in Fig. 10 B. According to the stimulation of proton symport activity by salt stress previously described [8,9], accumulation ratios were higher when the cells were incubated in 1M NaCl.

Although Fps1p-mediated glycerol transport and glycerol H^+/symport are distinct issues, this result suggests that the presence of extra copies of *FPS1* influence either active transport activity or the correspondent gene expression/regulation.

3.10. Studies on passive diffusion

Fps1p, as a channel, in the absence of stress, should be opened. Once opened, it is not awkward to postulate that glycerol free entrance, measured as a first-order kinetic

component, *i.e.* passive diffusion, could be done through the channel. Nevertheless, whatever the strain or the growth or experimental conditions, passive diffusion Kd values indicate always a rather slow entry, which is not very characteristic of a channel mediated substrate entry. *Fps1p* is not a pore like channel, since it is not found in a tetramer-like structure like aquaporins [34]. It is a monomer, very similar to *E. coli*, GlpFp, which, although it is also not a pore-like channel, mediates glycerol diffusion at much higher rates than the Kd measured in *S. cerevisiae* [17]. This could indicate that Fps1p is less efficient, or else, less specialised than GlpF for mediating glycerol entry. The main function attributed to Fps1p [13,35] is to regulate glycerol retention in relation to turgor changes, and to close/open upon sudden osmotic up- or down-shock. Thus considering, the hypothesis formulated above becomes plausible. The first-order kinetic component of Eq. 1 should then correspond to Fps1p mediated passive diffusion. This idea, however, is in conflict with the widely spread concept of glycerol being a liposoluble component permeating freely through yeast plasma membrane [6,7].

To test this hypothesis, we measured glycerol passive diffusion in cells incubated in ethanol. Ethanol has often been used as a membrane solubiliser [36]. Its primary target is plasma membrane, and passive diffusion has been shown to be exponentially enhanced by ethanol [37,38]. On the other hand, ethanol is generally an inhibitor of protein mediated transport [39], helping to destabilise protein functional insertion in the membrane and/or acting as an uncoupler [39,40,41]. Therefore, glycerol passive diffusion was measured again, using *fps1,* and the double mutant *fps1/yfl054c*, as well as W303-1A for control, cultivated in YEPD and incubated in buffer with ethanol (Fig 11). Ethanol concentration used was rather high. It was chosen after preliminary

experiments, which showed that lower ethanol concentrations did not induce significant and reproducible changes in glycerol entry.

Surprisingly, the results clearly show an increase in glycerol entry in the parental strain, and in *fps1* mutant (Fig 11). Kd values measured on wild type cells were ± 0.009 and ± 0.024 l h^{-1} g^{-1} dry weight (≈ 3 fold), respectively in the absence and in the presence of ethanol. On *fps1*, Kd changed from ± 0.0024 to ± 0.005 l h^{-1} g^{-1} dry weight (≈ 2 fold). In the double mutant, instead, no ethanol-induced changes were observed. Furthermore, Kd was identical to the one measured in *fps1* mutant: 0.0025 ± 0.0002 l h^{-1} g^{-1} dry weight (n=4).

This result, in what concerns ethanol action, is apparently not compatible with the hypothesis of glycerol passive entry being mediated by the channel. This, as a protein-mediated uptake, should not be inhibited and not stimulated by it. Nevertheless, to our knowledge, it is not known the effect ethanol might have on transport mediated by channels, which leaves us with a difficulty in interpreting results in this context. Results can though be interpreted in another perspective. In *fps1* mutants, the remaining glycerol entry was ≈ 30% the wt strain, and still prone to ethanol stimulation. On the other hand, the double mutant *fps1/yfl054c* was not affected by ethanol (Fig. 11). The difference between the two strains corresponds, obviously, to the presence of *YFL054c*, which transcription level is unknown. It is the first time that a phenotype can be attributed to *YFL054c*. This ORF encodes a putative channel-like protein [12], very similar to Fps1p. Apparently it does not mediate glycerol uptake under standard conditions, since kinetics are similar in *fps1* and *fps1/yfl054c* (Fig. 11). But, once in the presence of ethanol it presented passive diffusion, ≈ 2 fold higher than *fps1/yfl054c*. On the other hand, the wild type strain, having both genes, was stimulated ≈ 3 fold. This

suggests that the two channels have different physico-chemical/functional characteristics and thus are probably used for different purposes, what could explain why it has not been attributed a phenotype yet.

At this point we can state that glycerol passive diffusion is made mainly through Fps1p. Putative Yfl054cp channel-like protein, mediated glycerol passive diffusion only in the presence of very high ethanol concentrations. The remaining 30% of glycerol entry as first order kinetics, might be also mediated, since it was not affected by ethanol.

4. Discussion

4.1. Kinetic characteristics of Fps1p-mediated glycerol uptake

The results presented in this article aim to clarify the physiological role of Fps1p channel in what regards its ability to allow glycerol to permeate into the cell in the absence of salt- osmotic-stress or turgor changes. This entry has been classified as a *facilitated diffusion*, a transport obeying Michaelis-Menten kinetics, though not concentrative. Results have been presented before, suggesting that *fps1* mutant strain did not present this saturable glycerol uptake [11,10]. In spite of being an unusual member of the MIP gene family [13,14], Fps1p was proved to act as a glycerol channel, and ever since been known as the *yeast glycerol facilitator*. This classification does not gather a consensus opinion in the scientific community dedicated to transport biochemistry, but, is has been used to mean that the substrate can cross the membrane through this intrinsic protein in a fast and controlled manner yet not totally clarified.

Our results contradict published ones [11,10]. We show that both parental strain and *fps1* mutant present the same type of saturable kinetics, provided they are collected in the same growth phase. This way, glycerol permeabilisation into the cell through Fps1p and the so-called *facilitated diffusion* are different issues. As are also different issues, the channel activity and the active transport activity in derepressed cells, as had been suggested before [11,8,10,18]. Fps1p role keeps being the one of a channel, according to M. Tamás and co-workers [13], devoted to the main role of controlling glycerol efflux when the cell suffers a sudden osmotic shock or in response to turgor changes [35]. The problem rises trying to explain how the channel works in the absence of stress.

Glycerol kinase activity has been suggested to play an important role in the determination of labelled glycerol initial uptake rates, being able to create uptake artefacts, which suggest low affinity mediated transport. This is the case of *E. coli* glycerol transport system through the GlpF channel. The illusive existence of a facilitated diffusion was the consequence of a fast glycerol *catch* by the enzyme and subsequent saturation [42]. As a glycerol channel, it mediates the substrate entry into the cell at a flow rate of ± $2x10^6$ molecules sec^{-1} per cell (≈ 1μmol min^{-1} mg cell protein) [17]. It was more recently described as a non-saturable pore-like channel [43]. In *S. cerevisiae* the involvement of the first reaction of glycerol metabolism in glycerol transport measurements, was suggested to justify residual uptake of low Vmax, in mutants defective in *GUP1* and *GUP2* genes, both putative glycerol active transporters [18]. Furthermore, a channel/pore-like protein should allow transport of ± 10^7 molecules sec^{-1}, while permeases allowing equilibrating facilitated diffusion characterised so far, allow transport with Vmax ≤ 10^5 molecules sec^{-1} [44]. Active transporters generally present lower Vmax, 10 -10^3 molecules sec^{-1} [44]. Fps1p glycerol uptake rates [10,13] are far too low to be compatible with either, which is an argument more in favour of the channel being specialised in glycerol efflux rather than influx [13,35].

We observed that both wild type and *fps1* cells, grown on YEPD and collected during diauxic-shift, presented considerable levels of glycerol kinase activity. This is in accordance with more recent results on *GUT1* regulation [45]. Glycerol kinase activity levels were higher in *fps1* mutants than in wild type cells. Northern analysis of *FPS1* gene in wild type cells revealed, as predicted from the microarray data [33], an approximately constant level along growth curve in YEPD with very slight changes

during diauxic-shift. For this reason one could doubt that *facilitated diffusion* detection could be caused by Fps1p activity. Thus considering, we hypothesised again that kinase activity might be responsible for the uptake saturation kinetics. This was confirmed by the results obtained using *gut1* and *gut2* mutants grown on YEPD and collected in diauxic-shift. Clearly, *GUT1* interruption led to the disappearance of the saturation kinetics, while it was still present in *gut2* mutants. In cells grown in YEPE the active transport and the passive diffusion maintained identical values as in wild type cells. Nevertheless, as expected from previous results [18], in *gut1* strain, active transport Vmax is somewhat smaller than in *gut2*. Accordingly, in *gut1* strain maximum accumulation ratios were shown to correspond to the true strength of the transporter to create a chemical gradient, without the artefacts created by metabolism, since all radiolabel was expelled after *cold* glycerol addition.

Therefore, our results strongly suggest that glycerol *facilitated diffusion* is a Gut1p dependent experimental artefact, and glucose growing cells only present passive diffusion. Fps1p should thus not be considered a *glycerol facilitator*, in the sense that it does not mediate glycerol facilitated diffusion.

4.2. Fps1p mediates the major part of glycerol passive diffusion

Glycerol enters glucose-repressed cells by first-order kinetics, which at first hand, should indicate passive diffusion through the lipid bilayer. Nevertheless, we propose this might be a misconception, since the chemical properties of glycerol are not compatible with free passage through plasma membrane.

Glycerol oil/octanol partition coefficient (log P), according to Chemical Reviews [46] is –4.15. Comparing this value with the one of an obviously liposoluble component

like ethanol, which logP is –1.33, we can see that glycerol should not penetrate easily a lipid environment. Actually, sugars, which supposedly are not able to cross yeast plasma membrane through the lipid bilayer, have logP values lower than glycerol, like, for example, -1.96 for glucose and -1.77 for fructose [46] (Index Merck [47] simply states that glycerol is insoluble in oils). According to R.B. Gennis [48], glycerol permeability coefficient in egg phosphatidylcholine planar membranes is $\pm 10^{-6}$cm sec^{-1}, and $\pm 10^{-7}$cm sec^{-1} in red blood cells, corresponding to a passage through these membranes at a rate constant of $\pm 10^{7}$ sec^{-1}. This is 10^{3} times lower than the permeability coefficient and rate constant for water in the same membranes [44]. Thus considering, we could suppose that yeast plasma membrane should be, if not completely, almost impermeable to glycerol. Nevertheless, a plasma membrane is obviously a very complex and dynamic system and its physico-chemical properties cannot be resumed by partition or permeability coefficients.

The similarity of Kd values when determined in glucose-, ethanol- or glycerol-grown cells [8,9,10,18], which should have different lipid composition, supports this idea and suggests that Fps1p, once opened, should be responsible for the permeation of at least a part of glycerol *passive diffusion*. Moreover, Kd reduction observed under osmotic stress is consistent with the reduction observed in cell volume, due to the transient cell shrinkage upon osmotic shock. This comparison favours *passive diffusion* being done through the lipids rather than through a channel. Nevertheless, glycerol uptake, measured by M. Tamás and co-workers [13] in cells presenting Fps1p closed after being submitted to an osmotic shock, showed identical percentages of remaining first order kinetics glycerol uptake as cells in which the gene was interrupted. This way we can suggest that Fps1p is mediating the major part of the first order kinetics glycerol

entry according to changes in cell volume, which, in spite that Fps1p is not a mechanosensitive channel [12], is compatible with its ability to react to cell turgor changes [35]. Nevertheless, in *fps1* mutants, glycerol entry could still be measured: ≈ 0.1 mmol h^{-1} g^{-1} dry weight, around 10-20% uptake rates in wild type cells [13]. Also, a passive diffusion Kd could still be calculated: 0.004 ± 0.0002 l h^{-1} g^{-1} dry weight, around 60% the value measured in wild type cells [10]. The same way, these cells, subjected to 1 M NaCl shock, should have the channel closed [13] and thus, the non-saturable branch representing passive diffusion in the presence of salt we have measured ubiquitously [8,9,21] should not exist.

The remaining glycerol entry, although very slow and maybe almost not significant, can eventually be done across another channel, like, *e.g.* a less specific porin. This has been reported before for other biological systems [28,34,12,49]. We cannot disregard completely the hypothesis that at least a part of glycerol passive diffusion might occur through the lipids, eventually taking advantage of changes caused in the physico-chemical environmental conditioning. This corresponds to thermodynamic changes in lipid fluidity [50,51,37], or else, in membrane lipid organisation, which influences intrinsic proteins biochemical behaviour and molecular structure in a way yet poorly understood. Apparently, proteins undergo drastic structure and function changes under different environment rigor states [52]. The specific functional consequences are unclear. It is also not clear the role the lipid environment surrounding the protein might have on its activity regulation [44]. Furthermore, the lateral heterogeneity of the bilayer in terms of lipid composition and the localised interchanges it suffers by flip-flop movements, can create microcompartments in the membrane with specific vectorial properties. These can affect the orientation of

membrane components and thus protein insertion/activity as well as localised changes in lipid solubility properties for non-charged compounds [44]. Glycerol is one such case, and we have to admit that very little is known about its interaction with lipids.

Another result strengthened the concept of Fps1p-mediated passive diffusion: the fact that the inhibitory effect of CCCP over glycerol transport in *fps1* YEPE-grown cells was only visible at concentrations with a small contribution of passive diffusion to the bulk glycerol entry. Higher glycerol concentrations transport was insensitive to the drug action, showing, in comparison to wtild type, no transient progressive decrease in CCCP inhibition, correspondent to the presence of a two component glycerol uptake kinetics of the type of Eq. 1 as seen before [10].

On *S. cerevisiae*, former studies [53] have compared glycerol permeability using much different methodologies from ours, like gel permeation chromatography using [14C]ethylene glycol as internal standard. These suggested an inverse correlation between the molecular radius and permeation ability, *e.g.* being glycerol a small molecule with twice as much molecular radius as a water molecule, it should enter the cell at approximately half the speed. This reinforces the idea that glycerol free diffusion is most unlikely. Several attempts have been made to determine glycerol permeability coefficients in other biological systems. In the algae *Dunalliela salina*, in the cell envelope of pig erythrocytes and in egg phosphatidylcholine vesicles [54]; in the yeast *Debaryomyces hansenii* [55] as well as in *S. cerevisiae* as mentioned above [53,10]. Nevertheless, in all these cases, with the methodologies used, if glycerol was entering through any type of channel/porin, it would not have been possible to discriminate from lipid-through passive diffusion. Also, some attempts have been made as to determine glycerol permeability changes according to salt stress, and relate these with changes in

lipid composition. Apparently, only minor changes in the overall phospholipid fatty acid composition were found [53,10] and these could not be related with glycerol permeability. Maybe the only exception in the literature might be the case of the ergosterol-less nystatin-resistant mutants of the yeast *Candida albicans*, which glycerol permeability was considerably reduced in relation to wild type [56].

In our opinion, the concept of free entrance and leakage of glycerol in yeasts [6,7] has to be re-considered. Intracellular glycerol concentrations regulation might be tightly connected to the regulation of the entire glycerol pathway activity, important for several vital global regulation processes from cell metabolism. *FPS1* gene was first described as a suppressor of *fdp1* [16], allelic to *GGS1/TPS1* [11], a mutant unable to grow on fermentable carbon sources, presenting high glycerol intracellular concentrations [11]. The same suppression was obtained overexpressing *GPD1* gene, provided *FPS1* gene was present [11]. In either case, the production of high amounts of glycerol is favoured. An excess glycerol accumulation would thus most probably create turgor problems to the cell, which can be overcome by Fps1p export activity [34]. The authors suggested that *FPS1* might be important to control of cytosolic glycerol concentration through controlling the expression of *GPD1* an/or *GPD2* genes. But, in our opinion, this control could, in a much simpler way, be explained just by the channel function without having to postulate molecular relationships. On the other hand, glycerol production has been suggested to replace trehalose production for P_i supply in *ggs1/tps1* mutant, thus contributing for glycolysis to function [11,57]. This model fits the suppression phenotypes mentioned above. A. Blomberg [5] suggested that glycerol pathway could function as a futile cycle under stress. This way, glycerol cycle would (1) contribute to an P_i intracellular pool compatible with optimal glycolytic flux [57] and provide some

protection against oxidative stress through Gpp1p and Gpp2p activity [4], (2) provide cytosolic redox balance during glucose consumption mainly through Gpd1p activity [1,2] and coupling cytoplasmic to mitochondrial redox balance through the glycerol-3-phosphate shuttle [58], (3) interfere with lipid production through the regulation of glycerol-3-phosphate levels, (4) allow survival upon osmotic shock and growth at low a_w, increasing intracellular glycerol production through Gpd1p and Gpp2p activity [60,59,2,33,9,4], and reducing the speed of the upper part of glycolitic flux under stress [5] by deviating glucose for glycerol production. All this complexity centred in glycerol production/consumption is more logically supported by a tightly controlled transport across the plasma membrane.

Our results suggest that glycerol, according to its chemical properties, cannot permeate freely yeast lipid bilayer. They also demonstrate that passive diffusion is a mediated uptake, through Fps1p, together with other less specific channel/porin, not necessarily exclusively in response to a certain type of stress.

4.3. Overexpression of FPS1 *influence on H^+/glycerol symport*

An increase to the double was observed on Kd when extra copies of *FPS1* were expressing in a recipient cell. This is consistent with most of the diffusion being Fps1p-mediated. Higher intracellular glycerol concentration in *FPS1* overexpressing strains was also reported [11]. This is consistent with the higher H^+/symport Vmax observed in ethanol-growing cells. Repression over glycerol proton symport was probably alleviated, since some residual transport activity was detected in glucose-growing cells. This could be due to a number of reasons, involving expression regulation, either at the

molecular or biochemical levels. Eventually, the most straightforward cause would be an unbalance in intracellular glycerol levels, due to the increased leakage as a direct consequence of the outnumbered opened Fps1p (since these experiments were made in the absence of stress) with subsequent consequences in the carrier amount of protein. Active transport would thus compensate for increased leakage through more channel proteins.

4.4. A phenotype for FPS1 *homologous gene,* YFL054c

Finally, we should comment on the effect of ethanol on *FPS1* and *YFL054c* phenotypes. According to the literature [39], passage through the lipid bilayer is stimulated by ethanol, while protein mediated transports are inhibited. The fact that we had to raise ethanol concentrations up to 12% to be able to see some effect is consistent with glycerol passage not being done through the lipids, which would have been affected by much smaller amounts of ethanol [39,37]. On the other side, while water channels are often in a tetramer-like structure, glycerol channels apparently have been found to be monomers [34]. This could eventually be consistent with the resistance to ethanol de-stabilisation of the channel activity, also evidenced by the very high amounts of ethanol we had to use to see an effect.

Passive diffusion through Fps1p was confirmed, since the interruption of this gene reduced considerably Kd value. Further interruption of *YFL054c* did not change Kd value, which was identical in the simple and the double mutants. But in the presence of ethanol, both gene products activity were affected. *YFL054c* product (*fps1* mutants), reacted to ethanol in a similar manner as wild type strain, though not so strongly. Fps1p has been extensively studied in what relates cell response to osmotic shock and turgor

changes, while its homologue, to our knowledge, never presented phenotypes related to these type of stresses. Our results stress that, in spite that *YFL054c* phenotype was a first order kinetics glycerol uptake equal to Fps1p-mediated passive diffusion these two genes must perform different tasks, since this new phenotype was only detectable in the presence of high ethanol concentrations

We do not have an explanation for this difference. Ethanol, as a consequence of the changes in fluidity [40] acts also as an uncoupler [41]. Certain channels are gated by protons, like, for example the aquaglycerol porin (AQ3) from red blood cells [49] One such behaviour could explain the appearance of glycerol passive diffusion mediated by Yfl054cp in the presence of ethanol. This is the first time that a phenotype can be attributed to the absence of the gene *YFL054c*, which might be serving different purposes than *FPS1*. Taking into consideration the multiple and central roles glycerol plays in the cell metabolism this is not an improbable suggestion.

Acknowledgements

This work was supported by EC contract PL 950161 from BIOTECH - Cell Factories Program. We thank Markus M. Tamás, from the Dep. of Cell and Molecular Biology/Microbiology, Göteborg University, Sweden, for controlling *fps1*mutant strains integrity by using the survival test in the presence of antimonite and arsenite according to R. Wisocki and collaborators [23]. We also thank Bernard Prior (Orange Free State Univ., South Africa), Stefan Hohmann (Göteborg Univ., Sweden), Johan Thevelein (Leuven Catholic Univ., Belgium) and Morten Kielland–Brandt (Carlsberg Inst.,

Denmark), for the lively discussions which enabled to shade some light to such intricate results.

References

1. J. Albertyn, S. Hohmann, B. Prior, Characterization of the osmotic-stress response in *Saccharomyces cerevisiae:* osmotic stress and glucose repression regulate glycerol-3-phosphate independently, Curr. Genet. 25 (1994) 12-18.

2. R. Ansell, K. Granath, S. Hohmann, J.M. Thevelein, L. Adler, The two isoenzymes for yeast NAD^+-dependent glycerol 3-phosphate dehydrogenase encoded by GPD1 and GPD2 have distinct roles in osmoadaptation and redox regulation, EMBO J. 16 (1997) 2179-2187.

3. M. Rep, J. Albertyn, J.M. Thevelein, B.A. Prior, S. Hohmann, Different signalling pathways contribute to the control of GPD1 gene expression by osmotic stress in *Saccharomyces cerevisiae*, Microbiol. 145 (1999) 715-727.

4. A.K. Påhlman, K. Granath, R. Ansell, S. Hohmann, L. Adler, The yeast glycerol 3-phosphatases Gpp1p and Gpp2p are required for glycerol biosynthesis and differentially involved in the cellular responses to osmotic, anaerobic, and oxidative stress, J. Biol. Chem. 276 (2001) 3555-3563.

5. A. Blomberg, Metabolic surprises in *Saccharomyces cerevisiae* during adaptation to saline conditions: questions, some answers and a model, FEMS Microbiol. Lett. 182 (2000) 1-8.

6. O.F. Heredia, A. Sols, G. Delafuente, Specificity of the constitutive hexose transport in yeast, Eur. J. Biochem. 6 (1968) 321-328.

7. C. Gancedo, J.M. Gancedo, A. Sols, Glycerol metabolism by yeasts. Pathways of utilization and production, Eur. J. Biochem. 5 (1968) 165-172.

8. F. Lages, C. Lucas, Physiological characterisation of mediated glycerol uptake in *Saccharomyces cerevisiae*, Bioquim. Biophys. Acta 1322 (1997) 8-18.

9. F. Lages, M. Silva-Graça, C. Lucas, Active glycerol uptake is a mechanism underlying halotolerance in yeasts: a study of 42 species, Microbiol. 145 (1999) 2577-85.

10. F.C.W. F. Sutherland, F. Lages, C. Lucas, C. Luyten, J. Albertyn, S. Hohmann, B. Prior, S.G. Kilian, Characteristics of Fps1p-dependent and independent glycerol transport in *Saccharomyces cerevisiae,* J. Bacteriol. 179 (1997) 7790-7795.

11. K. Luyten, J. Albertyn, W. Skibbe, B. Prior, J. Thevelein, S. Hohmann, Fps1, a yeast member of the MIP family of channel proteins, is a facilitator for glycerol uptake and efflux and it is inactive under osmotic stress, EMBO J. 14 (1995) 1360-1371.

12. M. Bonhivers, J. M. Carbrey, S. J. Gould, P. Agre, Aquaporins in *Saccharomyces*. Genetic and functional distinctions between laboratory and wild-type strains, J. Biol. Chem. 273 (1998) 27565-27572.

13. M.J. M. Tamás, K. Luyten, C. F. Sutherland, A. Hernadez, J. Albertyn, H. Valadi, H. Li, B.A. Prior, S. Killian, J. Ramos, L. Gustafsson, J.M. Thevelein, S. Hohmann, Fps1p controls the accumulation and the release of the compatible solute glycerol in yeast osmoregulation, Mol. Microbiol. 31 (1999) 1087-1104.

14. R.M. Bill, K. Hedfalk, S. Karlgren, J.G.L. Mullins, J. Rydström, S. Hohmann, Analysis of the pore of the unusual Major Intrinsic Protein channel, yeast Fps1p, J. Biol. Chem. 276 (39) (2001) 36543-49

15. B. André, An overview of membrane transport proteins in *Saccharomyces cerevisiae*, Yeast 11 (1995) 1575-1611.

16. L. van Aelst, S. Hohmann, F. Zimmermann, A. Jans, J. Thevelein, A yeast homologue of the bovine lens fibre MIP gene family complements the growth defect of a *Saccharomyces cerevisiae* mutant on fermentable sugars but not its defect in glucose-induced RAS-mediated cAMP signalling, EMBO J. 10 (1991) 2095-2104.

17. K. Heller, E. Lin, T. Wilson, Substrate specificity and transport properties of the glycerol facilitator of *Escherichia coli,* J. Bacteriol. 144 (1980) 274-278.

18. H. Rottenberg, The measument of membrane potential and ΔpH in cells, organelles, and vesicles, Meth. Enzymol. LV (55) (1979) 547-569.

19. P. de la Peña, F. Barros, S. Gascon, P.S. Lazo, S. Ramos, Effect of yeast killer toxin on sensitive cells of *Saccharomyces cerevisiae,* J. Biol. Chem. 256 (1981) 10420-10425.

20. F. Lages, C. Lucas, Characterization of a glycerol/H^+ symport in the halotolerant yeast *Pichia sorbitophila,* Yeast 11 (1995) 111-119.

21. B. Hölst, C. Lunde, F. Lages, R. Oliveira, C. Lucas, M.C. Kielland-Brandt, *GUP1* and its close homologue *GUP2*, encoding multimembrane-spanning proteins involved in active glycerol uptake in *Saccharomyces cerevisia,* Mol. Microbiol. 37 (2000) 108-124

22. L. Neves, R. Oliveira, C. Lucas, Metabolic flux response to salt-induced stress in the halotoleant yeast *Debaryomyces hansenii,* Microbiol. 143 (1997) 1133-1139.

23. R. Wysocki, C.C. Chéry, D. Wawrzycka, M. van Hulle, R. Cornellis, J.M. Thevelein, M.J. M. Tamás, The glycerol channel Fps1p mediates the uptake of arsenite and antimonite in *Saccharomyces cerevisiae*, Mol. Microbiol. 40 (2001) 1391-1401.

24. J. Sambrook, E.F. Fritsch, T. Manniatis, Molecular Cloning, A Laboratory Mannual. Cold Spring Harbour Lab. Presds, 2nd Ed. (1990)

25. F.M. Ausubel, B. Brent, R.E. Kingston, D.D. Moore, J.G. Seidman, J.A. Smith, K. Struhl, (Eds.) Current Protocols in Molecular Biology, Massachussets General Hospital/ Harvard University, (K. Janssen Ed.) Vols. 1-3, John Wiley & Sons, Inc., New York (1996).

26. R.A. Farrell Jr, RNA Methodologies. A Laboratory Guide for Isolation and Characterisation. (1996) (2nd Ed.) Academic Press, San Diego, USA

27. S. Hohmann, Osmotic stress signalling and osmoadaptation in yeasts, Microbiol. Mol Biol. Rev., 66 (2) (2002) 300-372

28. B. Norkrans, A. Kylin, Regulation of the potassium to sodium ratio and of the osmotic potential in relation to salt tolerance in yeasts, J. Bacteriol. 100 (1969) 836-845.

29. J.H. van Eck, B.A. Prior, E.V. Brandt, The water relations of growth and polyhydroxy alcohol production by ascomycetous yeasts, J. Gen. Microbiol. 139 (1993) 1047-1054.

30. C. Prista, A. Almagro, M.C. Loureiro-Dias, J. Ramos, Physiological basis for the high salt tolerance of *Debaryomyces hansenii*, Appl. Environm. Microbiol, 63 (1997) 4005-4009.

31. M. Silva-Graça, L. Neves, C. Lucas, Physiological studies of long-term adaptation to salt stress by the extreme halotolerant yeast *Candida versatilis (halophila),* Yeast FEMS Research (2002) In press.

32. G.F. Sprague, J.E. Cronan, Isolation and characterization of *Saccharomyces cerevisiae* mutants defective in glycerol metabolism, J. Bacteriol. 129 (1977) 1335-1342.

33. J.L. DeRisi, V,R. Iyer, P.O. Brown, Exploring the metabolic and genetic control of gene expression on a genomic scale, Science, 278 (5338) (1997) 680-86.

34. V. Lagrée, A. Froger, S. Deschamps, I. Pellerin, C. Delamarche, G. Bonnec, J. Gouranton, D. Thomas, J.-F. Hubert, Oligomerisation state of water channels and glycerol facilitators. Involvement of loop E, J. Biol. Chem. 273 (51) (1998) 33949-953.

35. M.J. M. Tamás, M. Rep, J.M. Thevelein, S. Hohmann, Stimulation of the yeast high osmolarity glycerol (HOG) pathway: evidence for a signal generated by a change in turgor rather than by water stress, FEBS Lett. 21, 472 (1) (2000) 159-65.

36. S.P. Salgueiro, I. Sá-Correia, J.M. Novais, Ethanol-induced leakage in *Saccharomyces cerevisiae*: kinetics and relationship of yeast ethanol tolerance and alcohol fermentation productivity, Appl. Environm. Microbiol. 54 (1988) 903-909.

37. C. Leão, N. van Uden, Effects of ethanol and other alkanols on passive proton influx in the yeast *Saccharomyces cerevisiae*, Biochim. Biophys. Acta 774 (1984) 43-48.

38. R.P. Jones, P.F. Greenfield, Specific and non-specific inhibitory effects of ethanol on yeast growth, Enzyme Microb Technol. 9 (1987) 334-338.

39. N. van Uden, Effects of alcohols on membrane transport in yeasts. In, Alcohol Toxicity in Yeasts and Bacteria. N. van Uden (Ed.), CRC Press, Boca Raton, FL (1989).

40. D. Lloyd, S. Morrell, H.N. Carlsen, H. Degn, P.E. James, C.C. Rowlands, Effects of growth with ethanol on fermentation and membrane fluidity of *Saccharomyces cerevisiae*, Yeast 9 (1993) 825-833.

41. M.C. Loureiro-Dias, Santos, H., Effets of ethanol on *Saccharomyces cerevisiae* as monitored by in vivo ^{31}P and ^{13}C nuclear magnetic resonance, Arch. Microbiol. 153 (1990) 384-391.

42. S. Hayashi, E.C.C. Lin, Capture of glycerol by cells of *Escherichia coli*, Biochim. Biophys. Acta 94 (1965) 479-487.

43. C. Maurel, J. Reizer, J.I. Schroeder, M.J. Chrispeels, M.H. Jr Saier, Functional characterization of the *Escherichia coli* glycerol facilitator, GlpF, in *Xenopus* oocytes, J. Biol. Chem. 269 (1994) 11869-72.

44. R.B. Gennis (Ed.), Biomembranes: Molecular structure and function, Springer-Vrelag, N.Y. 2nd Ed (1997).

45. M. Grauslund, J.M. Rønnow, Expression of *GUT1*, which encodes glycerol kinase in *Saccharomyces cerevisiae*, is controlled by the positive regulators Adr1p, Ino2p and Ino4p and the negative regulator Opi1p in a carbon source-dependent fashion, Nucl. Ac. Res. 27 (1999) 4391-98.

46. Leo, C. Hansch, D. Elkins, Partition coefficients and their uses, Chem. Rev. 71 (6) (1971) 525-616.

47. *Anonimous*, The Merk Index of Chemicals and Drugs (1979)

48. K. Ishibashi, M. Kuwahara, Y. Gu, Y. Kageyama, A. Tohsaka, F. Suzuki, F. Marumo, S. Sasaki, Cloning and functional expression of a new water channel abundantly expressed in the testis, permeable to water, glycerol and urea, J. Biol. Chem. 272 (33) (1997) 20782-86

49. T. Zeuthen, D.A. Klaerke, Transport of water and glycerol in Aquaporin 3 is gated by H^+, J. Biol. Chem. 274 (31) (1999) 21631-36.

50. P.L. Yeagle, Lipid regulation of cell membrane structure and function, FASEB J. 3 (1989) 1833-42.

51. J. Cerbón, V. Calderón, Changes in the compositional asymmetry of phospholipids associated to the increment in the membrane surface potential, Biochim. Biophys. Acta 1067 (1991) 139-144.

52. C.H. Luan, T.M. Parker, K.U. Prasad, D.W. Urry, Differential scanning calorimetric studies of NaCl effect on the inverse temperature transition of some elastin-based polytetra-, polypenta-, and polynonapeptides, Biopolymers 31 (5) (1991) 465-475.

53. R. Scherrer, L. Louden, P. Gerhadt, Porosity of yeast cell wall and membrane, J. Bacteriol 118 (2) (1974) 434-540.

54. F.F. Brown, I. Sussman, M. Avron, H. Degani, NMR studies of glycerol permeability in lipid vesicles, erythrocytes and the alga *Dunaliella*, Biochim. Biophys. Acta 690 (1982) 165-173.

55. Tunblad-Johanson, L. Andrè, L. Adler, The sterol and phospholipid composition of the salt-tolerant yeast *Debaryomyces hansenii*-grown at various concentrations of NaCl, Biochim. Biophys. Acta 921 (1987) 116-123.

56. M. Pesti, R. Novak, Decreased permeability of glycerol in an ergosterol-less mutant of *Candida albicans*, Acta Microbiol. 31 (1984) 81-84.

57. J.M. Thevelein, S. Hohmann, Trehalose synthase: guard to the gate of glycolysis in yeast?, TIBS 20 (1995) 3-10.

58. Larson, I.-L. Påhlmann, R. Ansell, M. Rigoulet, L. Adler, L. Gustafsson, The imortance of glycerol 3-phosphate shuttle during aerobic growth of Saccharomyces cerevisiae, Yeast 14 (1998) 347-357.

59. J. Albertyn, S. Hohmann, J. Thevelein, B. Prior, GPD1, which encodes glycerol-3-phosphate dehydrogenase, is essential for growth under osmotic stress in *Saccharomyces cerevisiae*, and its expression is regulated by high-osmolarity glycerol response pathway, Mol. Cell. Biology 14 (1994) 4135-4144.

60. A.D. Brown, Compatible solutes and extreme water stress in eukaryotic micro-organisms, Adv. Microb. Physiol. 17 (1977) 181-242.K. Ishibashi, M. Kuwahara, Y. Gu, Y. Kageyama, A. Tohsaka, F. Suzuki, F. Marumo, S. Sasaki, Cloning and functional expression of a new water channel abundantly expressed in the testis, permeable to water, glycerol and urea, J. Biol. Chem. 272 (33) (1997) 20782-86.

Legends

Fig. 1. Eadie-Hofstee plot of [^{14}C]glycerol initial uptake rates of IGC3507 YEPD-grown cells collected in diauxic-shift, assayed at 30°C, in Tris citrate buffer, at pH 5.0. *Insert:* Michaelis-Menten presentation of the same experimental results.

Fig. 2. (**A)** Increase in intracellular glycerol as a function of extracellular salt concentration in the growth medium. *S. cerevisiae* IGC3507 was cultivated on MM with 2% glucose w/o salt and transferred medium w/ salt and incubated at 30°C. Initial medium pH was adjusted to 4.5. (**B)** Variation of glycerol concentration achieved after 3 hours incubation as a function of external salt concentration.

Fig. 3. Michelis-Menten presentation of [^{14}C]glycerol initial uptake rates of IGC3507 YEPD-grown cells collected in diauxic-shift, assayed at 30°C, in Tris citrate buffer, at pH 5.0, in the absence of salt (●) and in the presence of 500mM (□) and 1M NaCl (●). ***Insert*****:** Variation of apparent Km of each assay with salt concentration in the assay buffer (correlation -0.998).

Fig.4. Extracellular concentrations of glucose, ethanol and glycerol as well as glycerol kinase activity, determined in IGC3507 cells cultivated in MM with 2% (w/v) glucose (**A**) and in YEPD (**B**), and collected in three different growth phases (arrows).

Fig 5. (A). Eadie-Hofstee plots of initial uptake rates of [^{14}C]glycerol initial uptake rates of YEPD-grown cells of W303-1A (**A**) and *fps1* mutant (W303-1A background) (**B**), collected in exponential growth phase (❍) and during diauxic-shift (●). Cells were assayed at 30°C, in Tris citrate buffer, at pH 5.0. Raw experimental data kinetic

constants for diauxic-shift cells were Km 27 and 12 mM, Vmax 179 and 102 μmol h^{-1} g^{-1} dry weight for, respectively, W303-1A and *fps1* mutant (W303-1A background). *Inserts:* Glycerol accumulation ratios of the same YEPD diauxic-shift collected cells (●), comparing with exponentially collected ethanol-growing cells (▣).

Fig. 6. Percentage of the ionophore CCCP inhibitory effect over [^{14}C]glycerol initial uptake rates in YEPE-grown cells of W303-1A (●) and *fps1* mutant (W303-1A background) (○). (100% - glycerol uptake rate determined w/o CCCP).

Fig. 7. Growth curves on YEPD at 30°C of *S. cerevisiae* W303-1A (●), *fps1* (◍) and *gut1* (W303-1A background) (○). Glycerol kinase activity in the same cells is shown (black, grey and white bars, respectively). Growth phases are defined according to glucose and ethanol extracellular concentrations. ***Insert***: Northern results of *FPS1* expression obtained in cells collected in the first and in the last growth phases, respectivelly, (A) [gluc] > 10g l^{-1} and (B) [etho] > 100 mM. Relative fluorescence intensity of both samples is shown.

Fig 8. Comparison of specific growth rate (μ_g) and lag-phase duration in YEPD-grown cells of W303-1A and the correspondent *fps1* mutant, at 30°C, in the absence (grey bars) and in the presence of 1M NaCl (black bars).

Fig. 9. Eadie-Hofstee plots of [^{14}C]glycerol initial uptake rates in *gut1* and *gut2* mutants (W303-1A background), grown on YEPD and collected during diauxic-shift **(A)** and grown on YEPE and collected during exponential growth **(B).** ***Inserts*:** Accumulation curves of the same cells in the absence (●) and in the presence of the ionophore CCCP (□). Efflux obtained using *cold* glycerol (○).

Fig 10. (A) Eadie-Hofstee plot of experimental data of [^{14}C]glycerol (●) initial uptake rates and glycerol-driven initial external alkalinization rates (■) of YSH294 cells-grown on MM with 0.2% glucose and 2% glycerol, collected in exponential growth phase and assayed at 30°C, in Tris citrate buffer, at pH 5.0. **(B)** Accumulation ratios of cells cultivated in MM with 2% glucose and collected in mid exponential phase: YHS294 (●,○) and IGC3507 (▲,△). Cells were assayed in the absence (●, ▲) and in the presence of 1M NaCl (○, △).

Fig. 11. Effect of 12% (v/v) ethanol shock on passive diffusion Kd on W303-1A (○,●) *fps1* (□,■) and *fps1/yfl054c* (△,▲). Absence of ethanol (white symbols) and presence (black symbols).

Table I
S. cerevisiae strains used in this work

Strain Designation	Genotype	Origin/Description
IGC 3507	wild type (diploid)	PYCC‡
W303-1A	*MAT*a *leu2-3, 112 ura3-1 trp1-1 his3-11, 15 ade2-1 can1-100*	Thomas and Rothstein (1989) #
YSH 6.36.-3B	*Matα leu 2-3/112 trp1-92 ura 3-52 GAL SUC mal0*	Hohmann *et al* (1993) #
---	W303-1A *fps1*::LEU2	Van Aelst *et al*. (1991) #
---	W303-1A *fps1*::LEU2 *YLF054c*::TRP1	Van Aelst *et al*. (1991) #
YSH 294	YSH 6.36.-3B yEplac195 FPS1::URA3	Luyten *et al*. (1995) #
---	W303-1A yEplac195 FPS1::URA3	Luyten *et al*. (1995) #
Cly1	W303-1A *gut1*Δ	Hölst *et al*. (2000) †
BHY40	W303-1A *gut2*Δ	Hölst *et al*. (2000) †

‡ Portuguese Yeast Culture Collection (New University of Lisbon, Portugal)
# S. Hohmann (Göteborg University, Sweden) and J. Thevelein (Katholieke Universiteit te Leuven, Belgium)
† Carlsberg Laboratory, Yeast Physiology Department, Copenhagen, Denmark

Table II

Kinetic parameters of initial uptake rates of [^{14}C] glycerol at 30°C and pH 5.0 in cells grown in glucose rich medium (YEPD) and harvested in exponential and post-diauxic growth phases.

Growth medium	YEPD					
Growth phase	Exponential			Diauxic- shift		
Strain	Km (mM)	Vmax (μmol h^{-1} g dwt^{-1})	Kd (l h^{-1} g d wt^{-1})	Km (mM)	Vmax (μmol h^{-1} g dwt^{-1})	Kd (l h^{-1} g d wt^{-1})
W303-1A	___	___	0.006 (1)	***5.0 ± 0.3***†	***100 ± 5***†	***0.013 ± 0.0004***†
fps1	___	___	0.003 (2)	6.2 (2)	104.5 (2)	0.005 (2)
gut1	___	___	0.010 (2)	___	___	0.009 (2)
gut2	n.d.	n.d.	n.d.	7.0 ± 7.5 (3)	94 ± 29 (3)	0.005 ± 0.001 (3)

† Sutherland *et al.* (1997)

___ No glycerol uptake saturation kinetics detected

n.d. Not determined

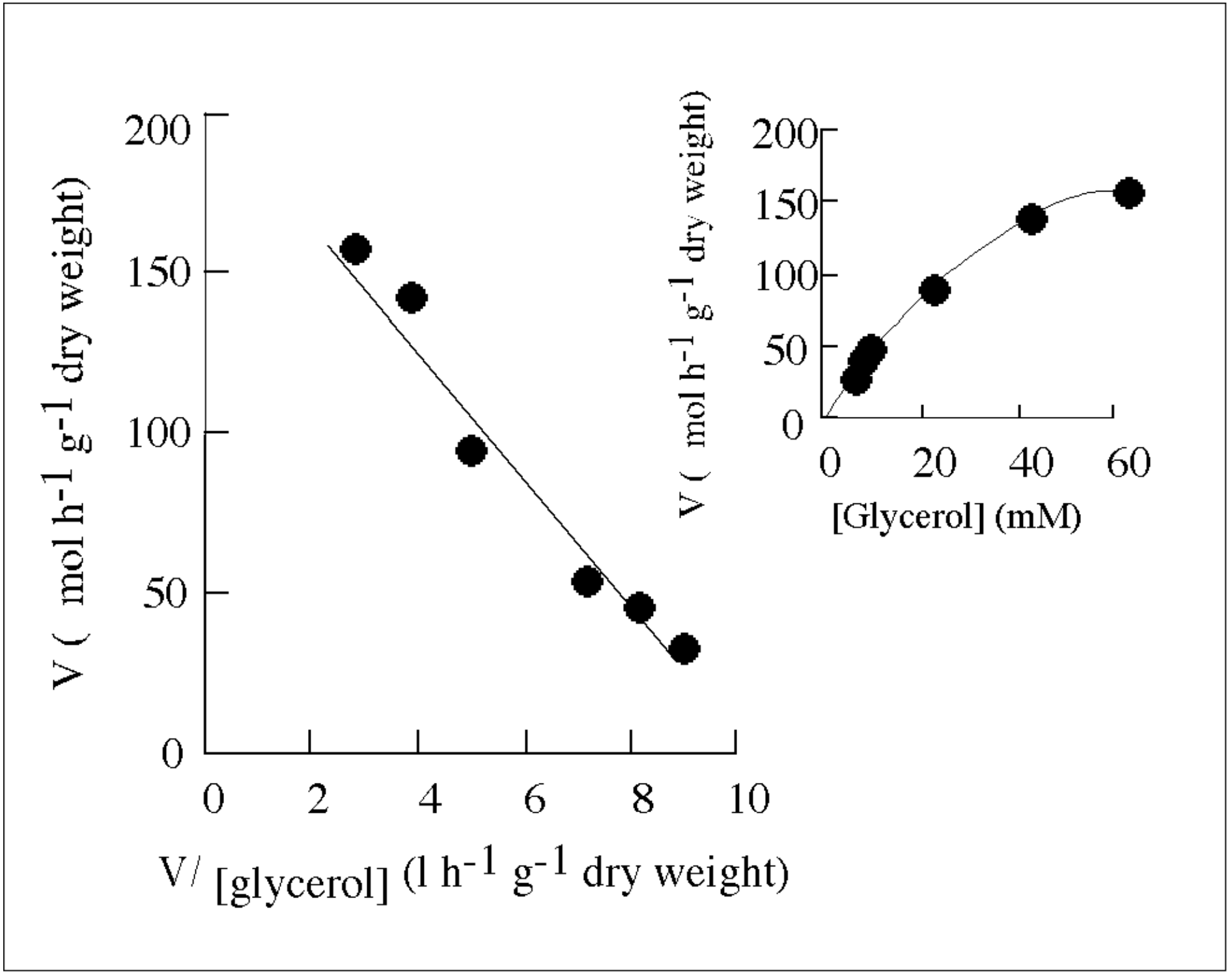
Table III

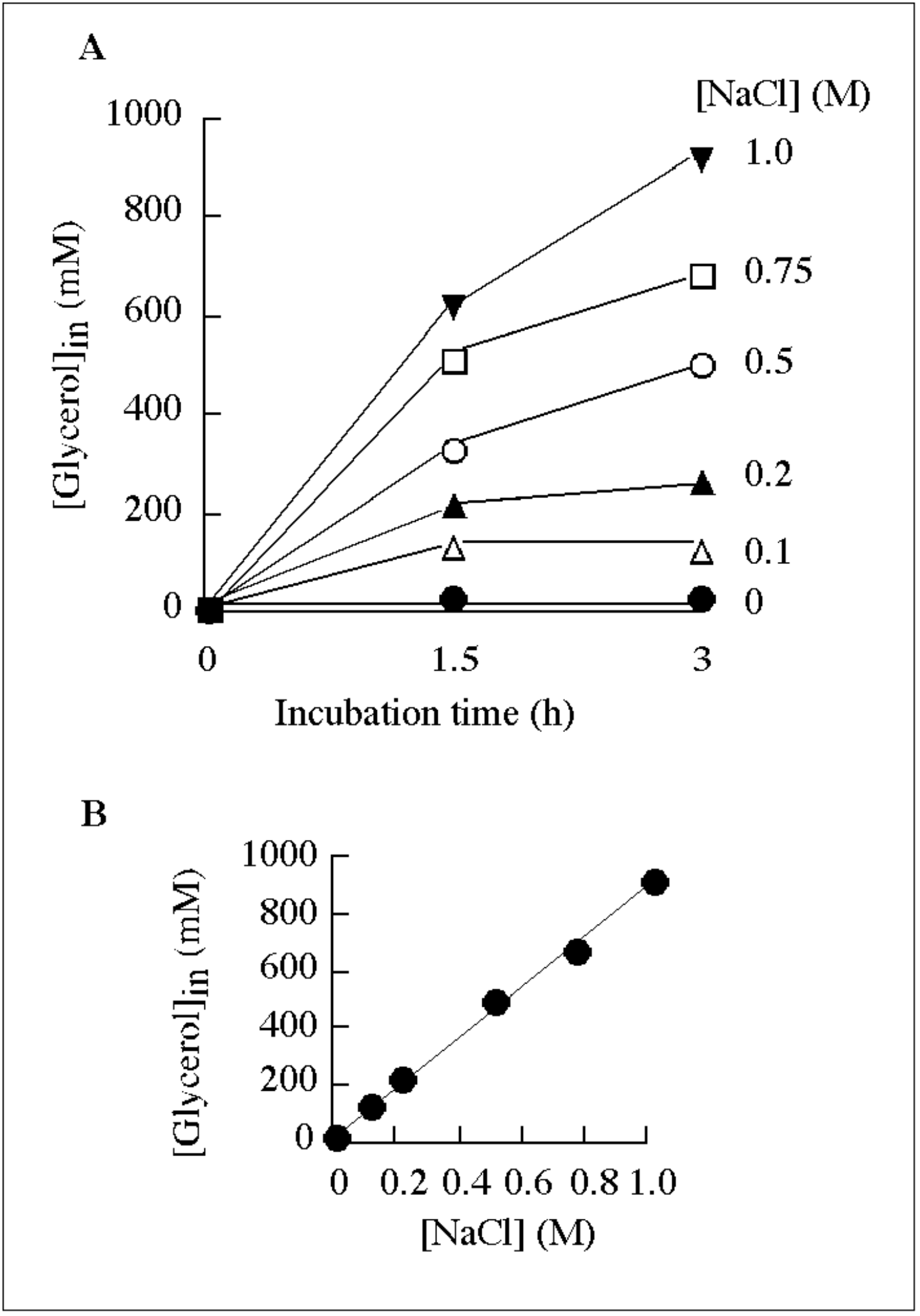
Glycerol uptake kinetic constants determined in *FPS1* overexpressed strains in two different genetic backgrounds grown on YEPE and collected in mid-exponential growth phase. Results obtained by computer regression anlysis.

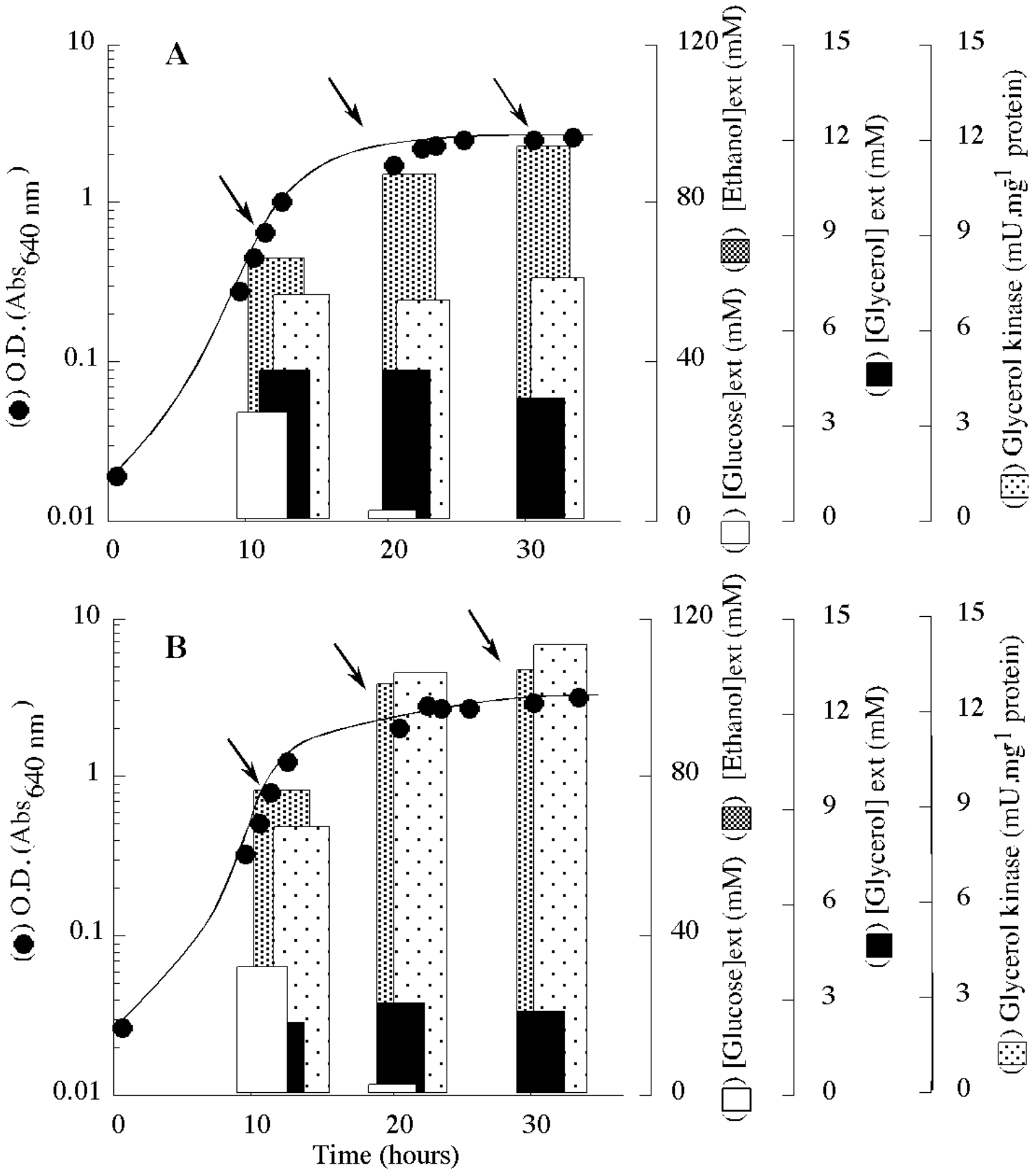
Strains \ Kinetic constants	[^{14}C] Glycerol Uptake		Glycerol Driven Proton Uptake		Passive Diffusion
	Km †	Vmax ‡	Km †	Vmax ‡	Kd #
YSH 6.36.-3B yEp lac195 FPS1::URA3	1.02 ± 0.09 (3)	651 ± 42 (3)	1.64 ± 1.06 (4)	996 ± 241 (4)	0.027 ± 0.006 (3)
W303-1A yEp lac195 FPS1::URA3	1.47 ± 0.52 (3)	519 ± 77 (3)	+	+	0.020± 0.002 (3)

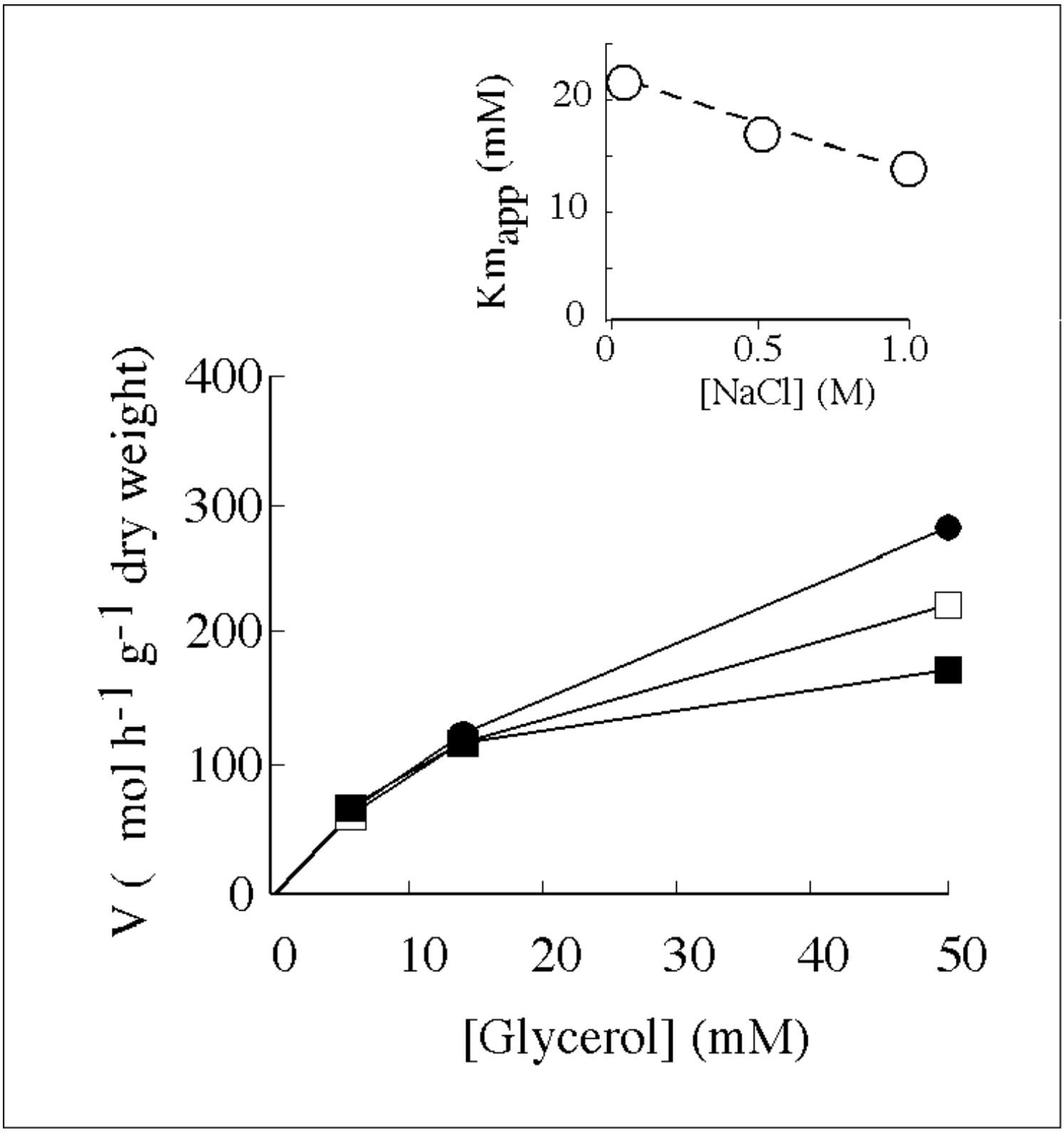
+ Presence of glycerol driven proton uptake (kinetic constants not determined)

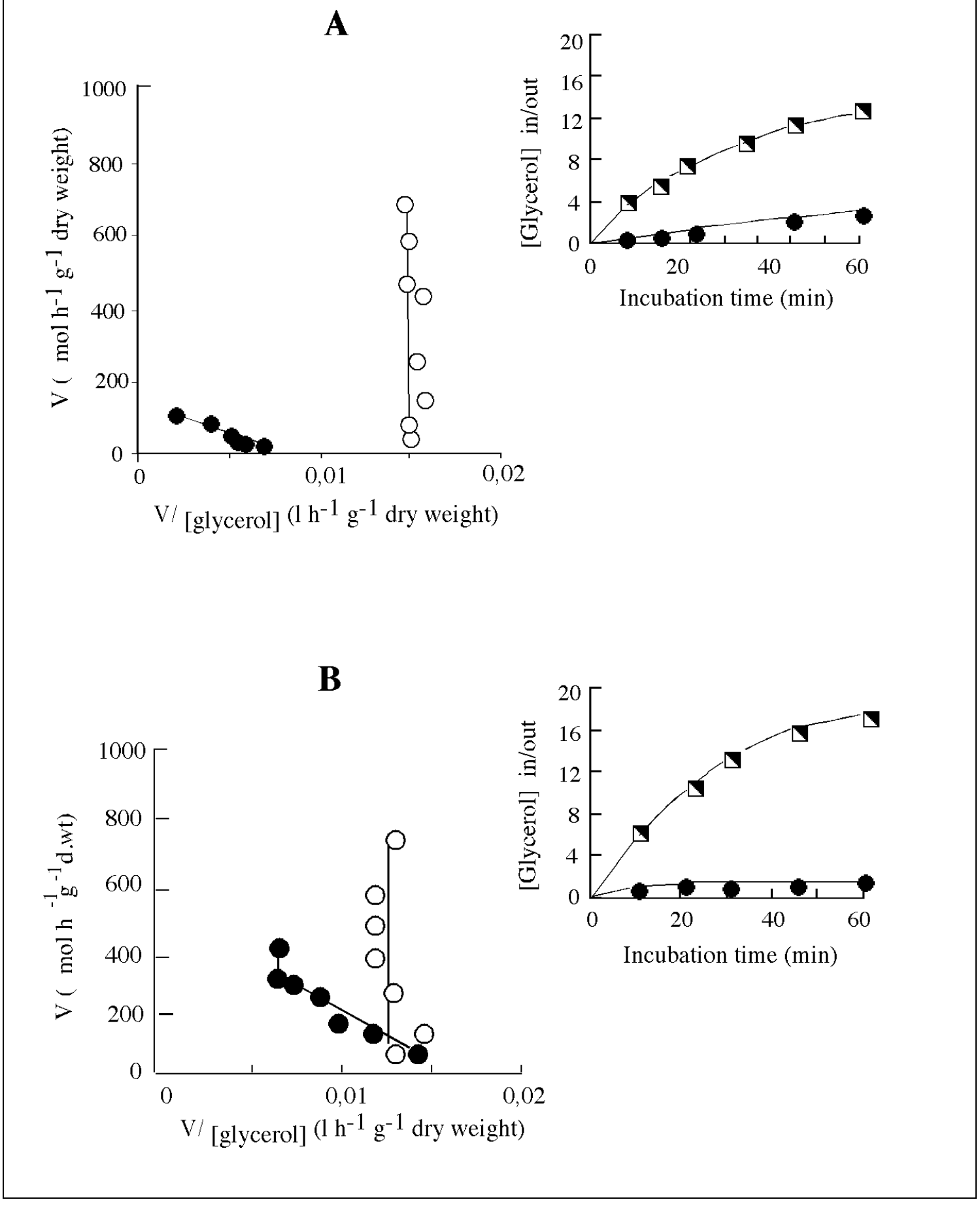
† (mM) ‡ (μmol h^{-1} g^{-1} d.wt.) # (l h^{-1} g^{-1} d.wt.)

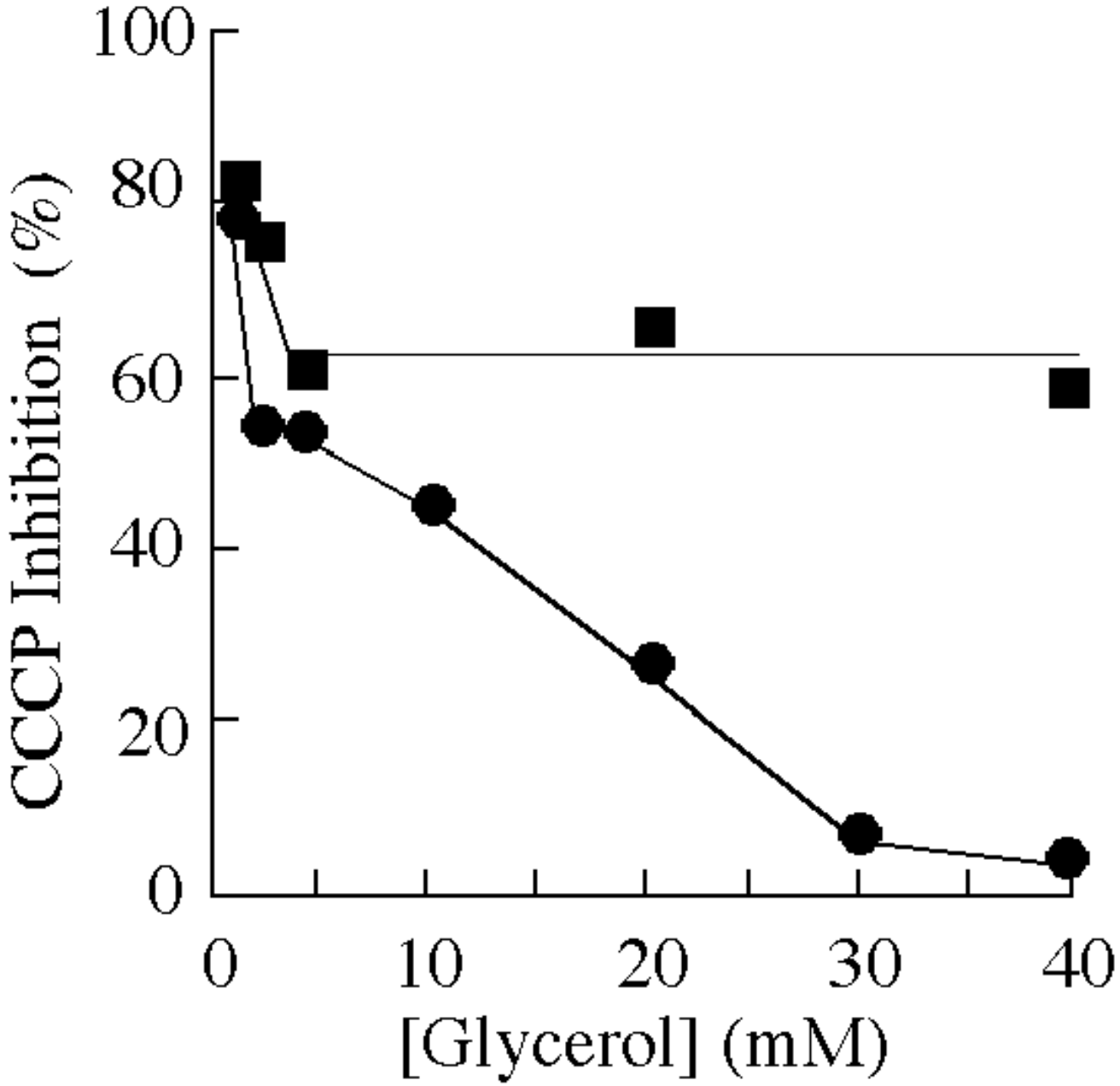

200
150
100
50
0
0
2
4
6
8
10
V (mol h-1 g-1 dry weight)
V/ [glycerol] (l h-1 g-1 dry weight)
V (mol h-1 g-1 dry weight)
200
150
100
50
0
0
20
40
60
[Glycerol] (mM)

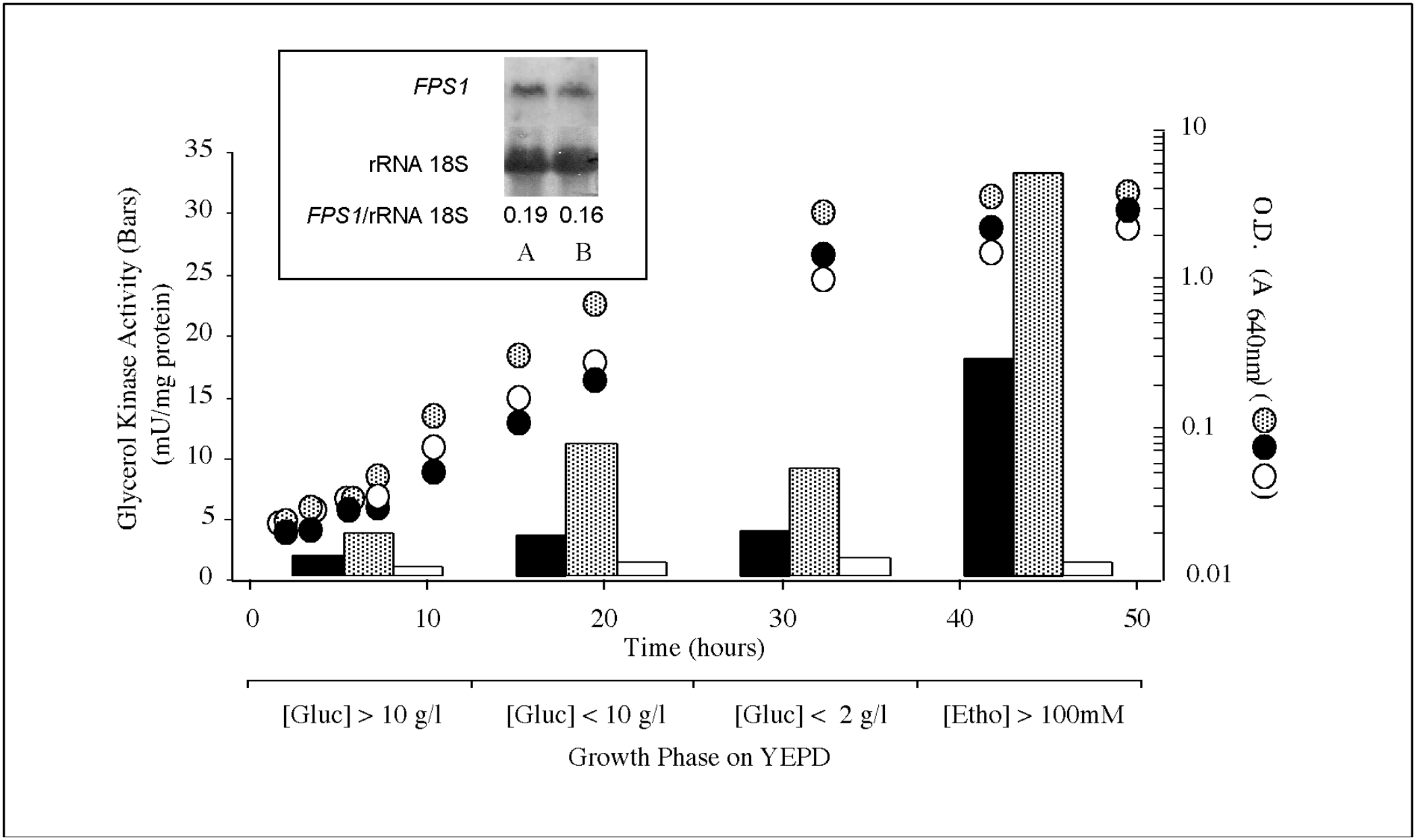

A
[NaCl] (M)
1.0
0.75
0.5
0.2
0.1
0
1000
800
600
400
200
0
[Glycerol]in (mM)
0
1.5
3
Incubation time (h)
B
1000
800
600
400
200
0
[Glycerol]in (mM)
0 0.2 0.4 0.6 0.8 1.0
[NaCl] (M)

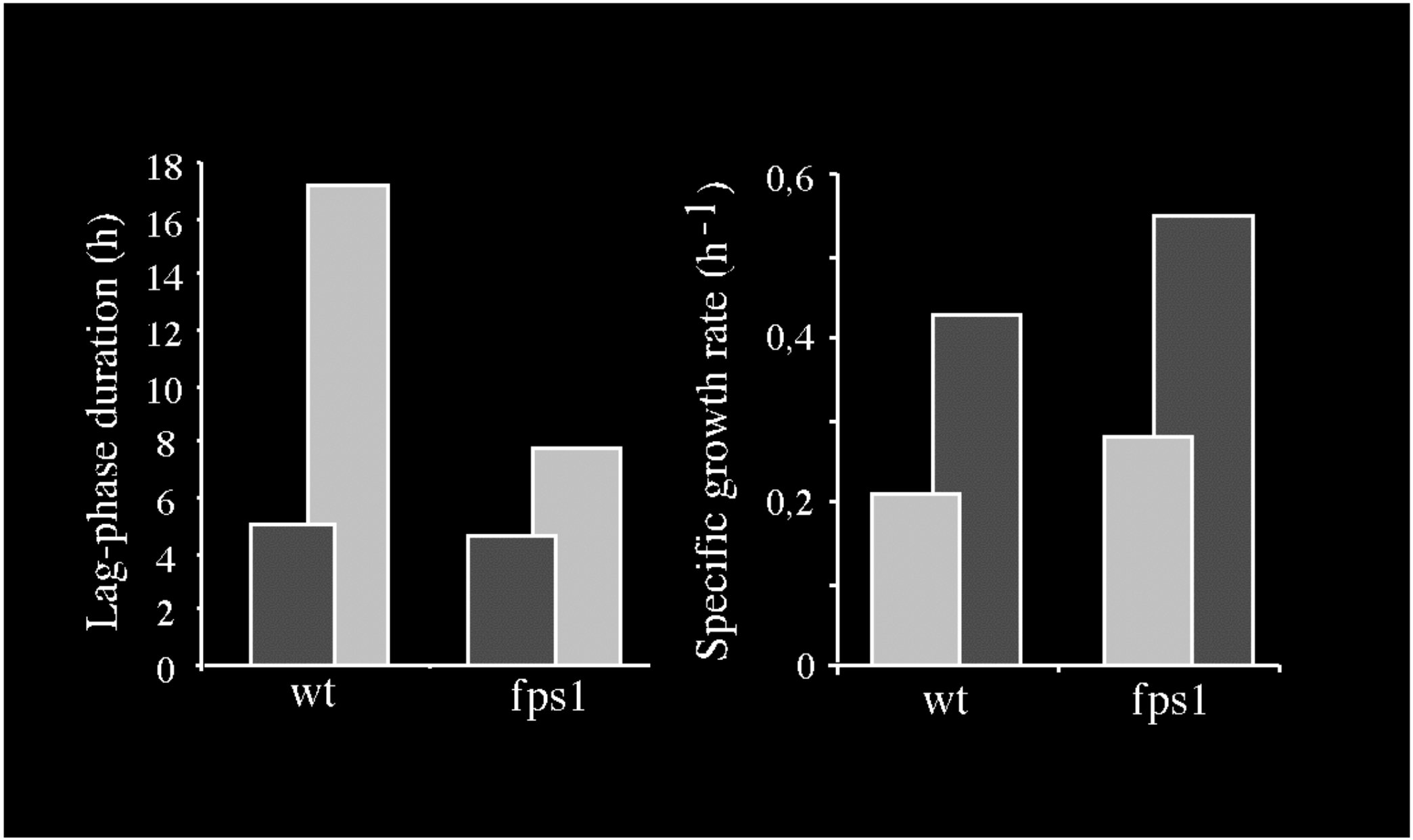

A
B
(●) O.D. (Abs640 nm)
(□) [Glucose]ext (mM)
(▩) [Ethanol]ext (mM)
(■) [Glycerol] ext (mM)
(⁝⁝) Glycerol kinase (mU.mg-1 protein)
Time (hours)
10
1
0.1
0.01
120
80
40
0
15
12
9
6
3
0
0
10
20
30

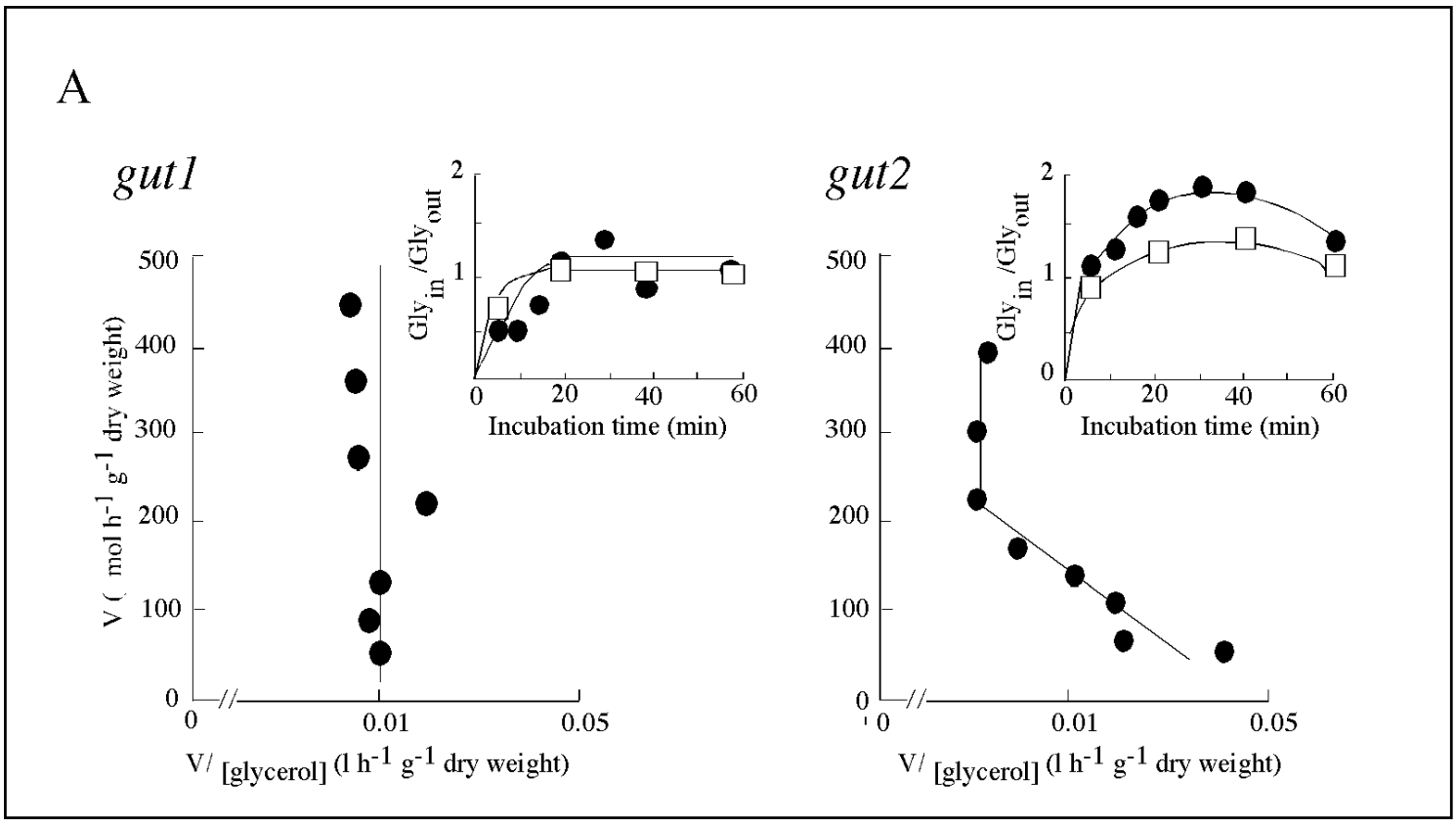

Km$_{app}$ (mM)
20
10
0
0
0.5
1.0
[NaCl] (M)
V (mol h^{-1} g^{-1} dry weight)
400
300
200
100
0
0
10
20
30
40
50
[Glycerol] (mM)

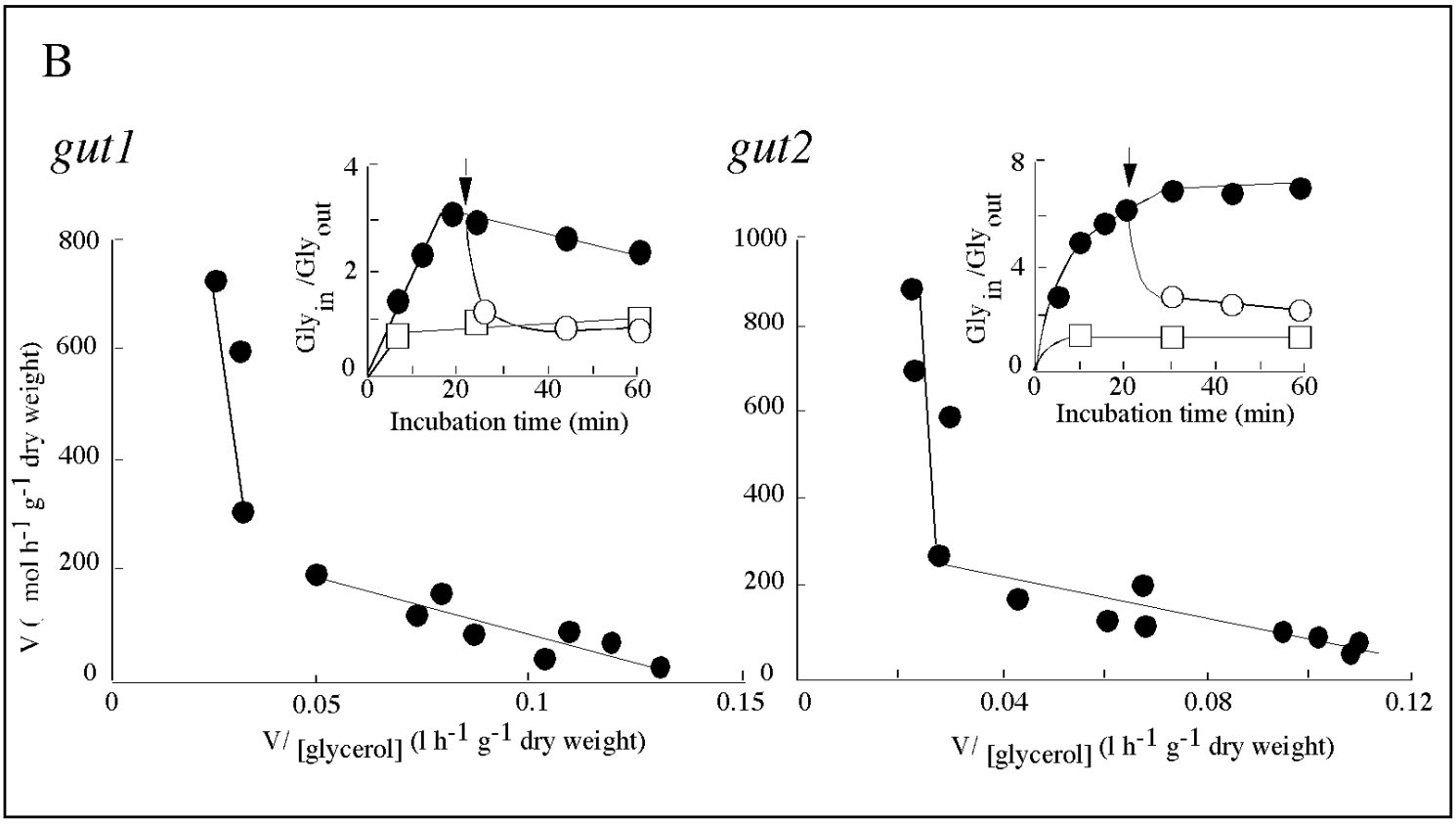

A
1000
800
600
400
200
0
V (mol h-1 g-1 dry weight)
0
0,01
0,02
V/ [glycerol] (l h-1 g-1 dry weight)
20
16
12
8
4
0
[Glycerol] in/out
0
20
40
60
Incubation time (min)
B
1000
800
600
400
200
0
V (mol h -1 g -1 d.wt)
0
0,01
0,02
V/ [glycerol] (l h-1 g-1 dry weight)
20
16
12
8
4
0
[Glycerol] in/out
0
20
40
60
Incubation time (min)

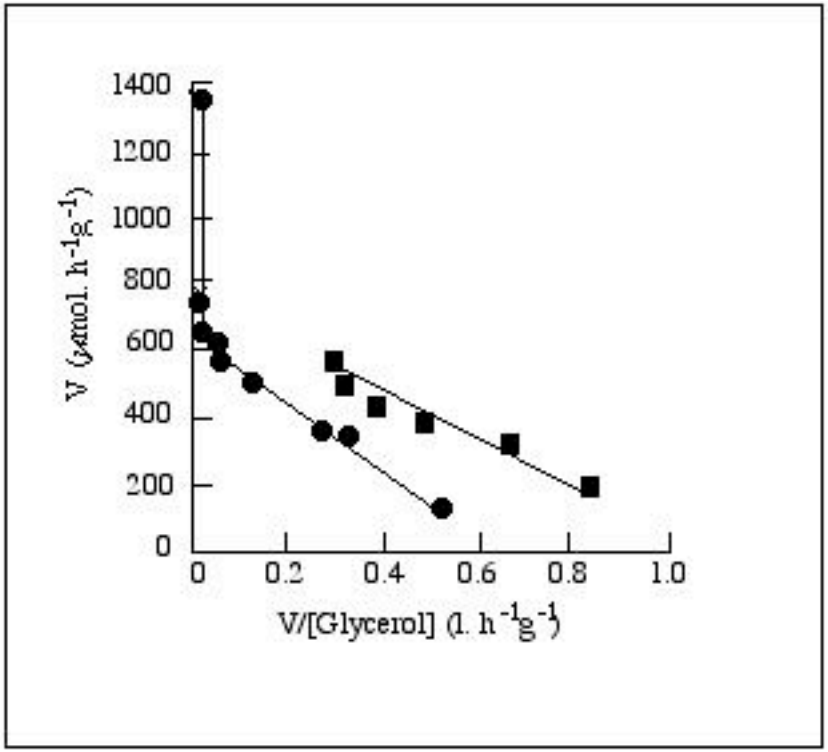

100
80
60
40
20
0
CCCP Inhibition (%)
0
10
20
30
40
[Glycerol] (mM)

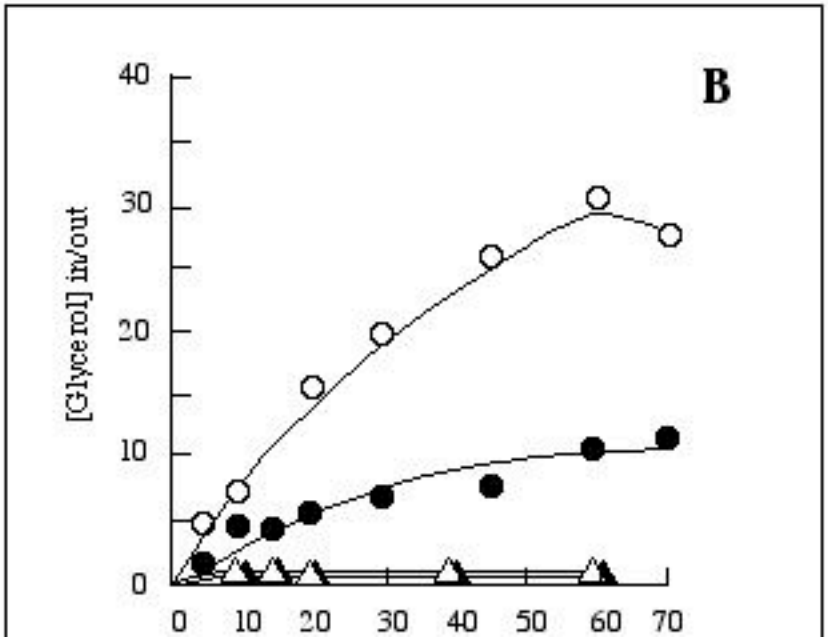

FPS1
rRNA 18S
FPS1/rRNA 18S
0.19 0.16
A B
Glycerol Kinase Activity (Bars)
(mU/mg protein)
35
30
25
20
15
10
5
0
10
1.0
0.1
0.01
O.D. (A 640nm) (●○)
0
10
20
30
40
50
Time (hours)
[Gluc] > 10 g/l
[Gluc] < 10 g/l
[Gluc] < 2 g/l
[Etho] > 100mM
Growth Phase on YEPD

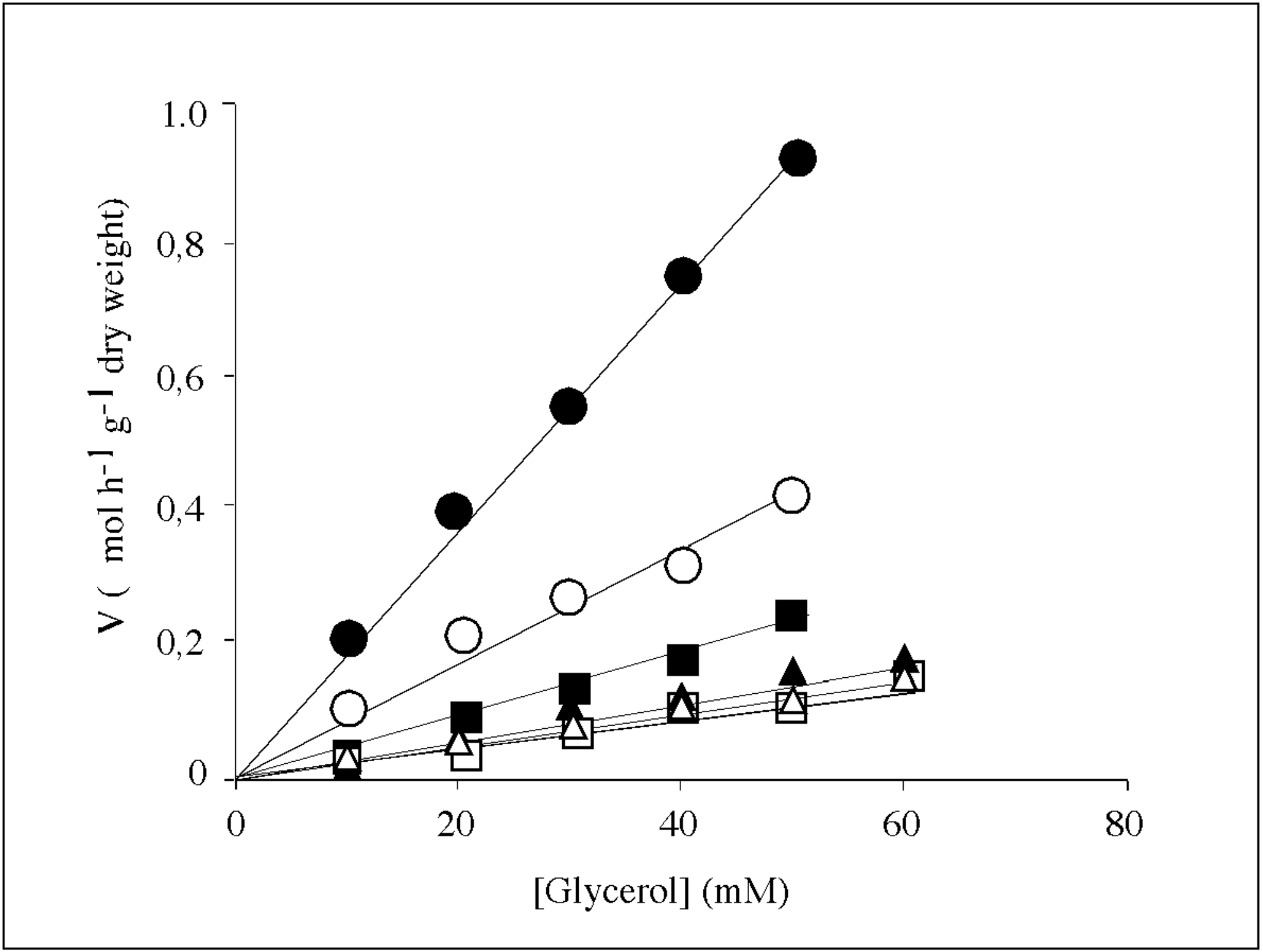

Lag-phase duration (h)
18
16
14
12
10
8
6
4
2
0
wt
fps1
Specific growth rate (h-1)
0,6
0,4
0,2
0
wt
fps1


A
gut1
gut2
V (mol h-1 g-1 dry weight)
V/ [glycerol] (l h-1 g-1 dry weight)
Gly in /Gly out
Incubation time (min)
0
100
200
300
400
500
0.01
0.05
20
40
60
1
2


B
gut1
gut2
V (mol h-1 g-1 dry weight)
V/ [glycerol] (l h-1 g-1 dry weight)
Gly in /Gly out
Incubation time (min)
0
200
400
600
800
1000
0.05
0.1
0.15
0.04
0.08
0.12
20
40
60
2
4
8


1400
1200
1000
800
600
400
200
0
V (μmol. h-1g-1)
0
0.2
0.4
0.6
0.8
1.0
V/[Glycerol] (l. h-1g-1)


B
40
30
20
10
0
[Glycerol] in/out
0
10
20
30
40
50
60
70


1.0
0,8
0,6
0,4
0,2
0
V (mol h-1 g-1 dry weight)
0
20
40
60
80
[Glycerol] (mM)